CYTOKINES AND THEIR SIGNALING IN CHRONIC INFLAMMATORY DISEASES AND BEYOND

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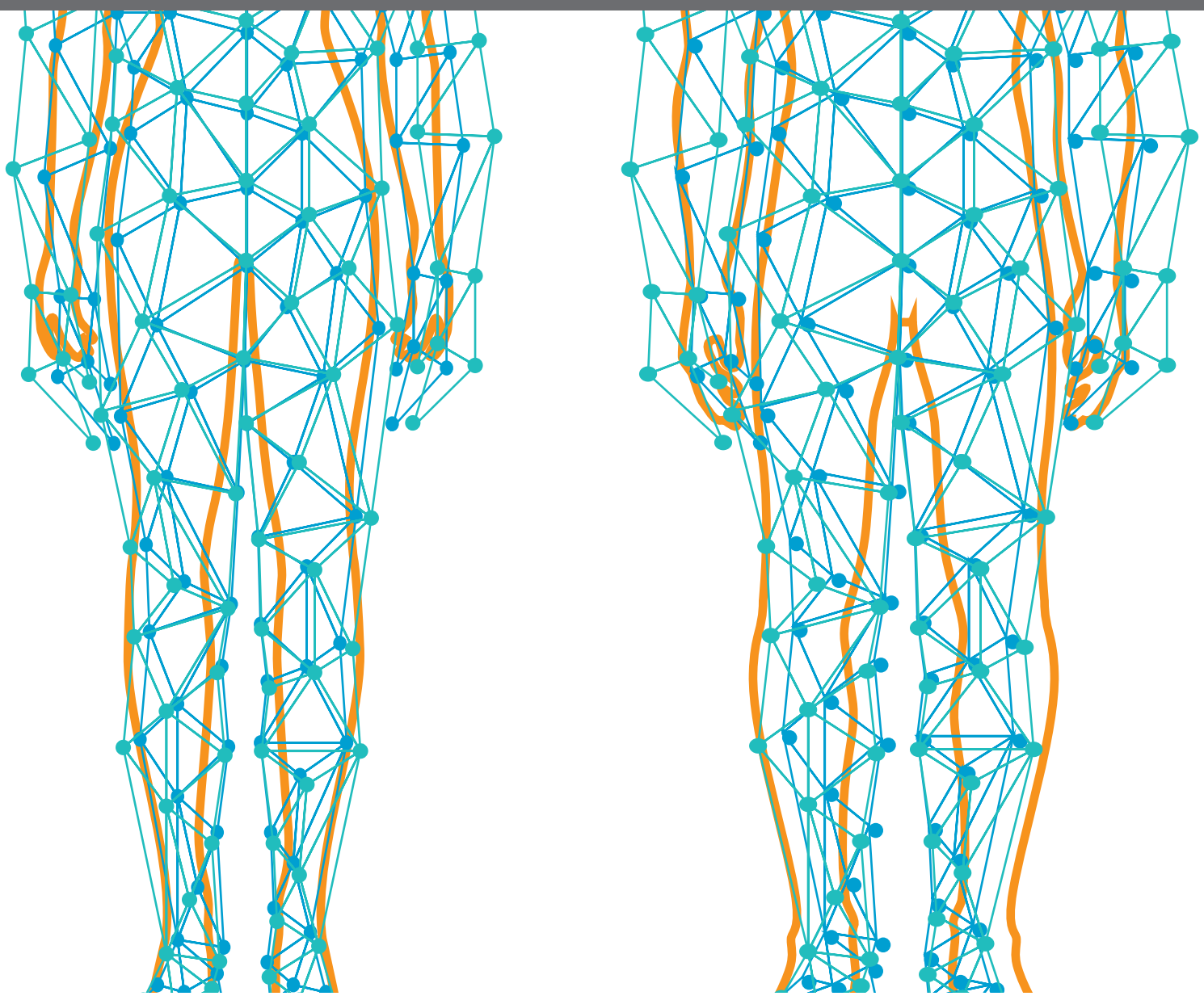

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CASE REPORT
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Case Report: A Case of Intestinal Behçet's Disease Exhibiting Enhanced Expression of IL-6 and Forkhead Box P3 mRNA After Treatment With Infliximab

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Behçet's disease (BD) is a rare inflammatory condition characterized by oral and genital ulcers, skin lesions, as well as ophthalmological, neurological, and gastrointestinal manifestations. BD involving the gastrointestinal tract is known as intestinal BD. The mucosa of the gastrointestinal tract of patients with intestinal BD exhibits enhanced levels of proinflammatory cytokines, such as IL-1β, IL-6, and TNF-α. These proinflammatory cytokines play pathogenic roles in the development of BD, as evidenced by the fact that biologics targeting these cytokines effectively induce BD remission. It should be noted, however, that the molecular mechanisms by which the blockade of these cytokines suppresses chronic inflammatory responses in BD are poorly understood. Herein, we report a case of intestinal BD resistant to prednisolone that was successfully treated with infliximab (IFX). The induction of remission by IFX was accompanied by a marked elevation of IL-6 and forkhead box P3 (FOXP3) at mRNA level. This case suggests that induction of remission by IFX is mediated not only by the suppression of TNF-α-mediated signaling pathways, but also by the promotion of IL-6 expression and accumulation of regulatory T cells expressing FOXP3.

Keywords: intestinal Behçet's disease, IL-6, Foxp3, infliximab, regulatory T cells

INTRODUCTION

Behçet's disease (BD) is a rare inflammatory condition characterized by oral and genital ulcers, skin lesions, and patients with BD exhibit ophthalmological, neurological, and gastrointestinal manifestations (1). Approximately 5–15% of patients with BD are diagnosed with intestinal BD, and they usually present with abdominal pain and diarrhea (1, 2). A typical endoscopic finding in patients with intestinal BD is a giant, oval-shaped, deep punched-out ulcer in the ileocecal area (1). The active phase of BD is characterized by high T helper 17 (Th17)/regulatory T cell (Treg) ratio (3, 4). In addition to the involvement of Th17 and Treg cells, increased levels of the proinflammatory cytokines, such as IL-1β, IL-6, and TNF-α, underlie the immunopathogenesis of BD, including that of intestinal

BD (1, 5). This idea is supported by the clinical success of biologics targeting proinflammatory cytokines in patients with BD (1, 5, 6). For example, administration of infliximab (IFX) and anakinra, which neutralize TNF-α and IL-1β, respectively, led to remission even in patients with BD refractory to prednisolone (PSL) (1, 5, 6). It should be noted, however, that the molecular mechanisms underlying the suppression of chronic inflammatory responses in intestinal BD are poorly understood. It is possible that the blockade of proinflammatory cytokines is not limited to the suppression of signaling pathways specific to each cytokine. Indeed, the neutralization of TNF-α by IFX or adalimumab does not always suppress TNF-α-mediated signaling pathways, but also promote the accumulation of Tregs expressing forkhead box P3 (FOXP3) (7, 8). Herein, we report a case of intestinal BD refractory to PSL that was successfully treated with IFX. The induction of remission by IFX in this case was accompanied by a marked elevation of IL-6 and FOXP3.

CASE DESCRIPTION

A 69-year old man was admitted for further examination of bloody diarrhea, appetite loss, and high-grade fever (>38°C), that persisted ∼1 month. He was diagnosed with uveitis and treated at the Department of Ophthalmology. On admission, he developed periodic fever accompanied by bloody diarrhea. Physical examination revealed oral aphthous ulcers without genital ulcers or erythema nodosum. Leukocytosis (white blood cell count: 10,200/μL) and anemia (hemoglobin: 9.1 g/dL) were detected in complete blood cell counts. Although, the liver and kidney function parameters were within normal limits, severe hypoalbuminemia (albumin: 1.7 g/dL, normal range 4.1–5.1) was seen. Serum concentrations of C-reactive protein (8.717 mg/dL, normal range <0.14) and IgG (2,492 mg/dL, normal range 861–1,747) were elevated. The patient's human leukocyte antigen was B54, and neither anti-neutrophil cytoplasmic antibody nor anti-nuclear antibody was positive. No pathogenic bacteria were detected in blood or stool cultures.

DIAGNOSTIC ASSESSMENT

Whole body computed tomography was performed to identify the causes of bloody diarrhea and periodic fever. Diffuse wall thickening of the ascending and transverse colon was detected on abdominal computed tomography. Deep, punched-out, oval-shaped, and semi-circumferential ulcers were observed in the cecum and sigmoid colon, respectively, during colonoscopy (**Figure 1A**). Pathological examination revealed destruction of the crypt architecture and massive accumulation of lymphocytes and granulocytes in the colonic lamina propria (**Figure 1B**). Granuloma formation or crypt abscesses were not observed. Polymerase chain reaction (PCR) tests for the detection of tuberculosis or cytomegalovirus using colonic biopsy samples were negative. These endoscopic and pathological findings were not consistent with Crohn's disease or ulcerative colitis. Based on the presence of uveitis, oral ulcers, and intestinal symptoms, the patient was diagnosed with intestinal BD according to the international diagnostic criteria for BD (2).

Colonic biopsy samples were subjected to immunohistochemical analysis, as previously described (9, 10). Briefly, deparaffinized sections were incubated with rabbit anti-human CD3 monoclonal antibody (mAb, clone 2GV6, Roche Diagnostics), mouse anti-human CD20 mAb (clone L26, Roche Diagnostics), rabbit anti-human polyclonal myeloperoxidase $(MPO)^+$ Ab (#518-114428, Roche Diagnostics) and mouse anti-human CD68 mAb (clone KP-1, Agilent Technology) followed by visualization of protein expression by Dako EnVision$^+$ System (Agilent Technology). Primary Ab dilution was done according to the recommendation of the manufacturers. Immunohistochemical photographs were captured by microscopy (Biozero, BZ-8100, Keyence). As shown in **Figure 1B**, the colonic mucosa was characterized by massive accumulation of $CD3^+$ T cells, $CD68^+$ macrophages, and MPO^+ granulocytes, but not of $CD20^+$ B cells (data not shown).

The initial treatment with oral colchicine (1.5 mg/day) based on the diagnosis of intestinal BD was unsuccessful. The patient was then treated with oral PSL (40 mg/day) with a tapering schedule as shown in the clinical course (**Figure 2A**), which again failed to improve his symptoms. As shown in **Figure 2B** (top panel), active ulcers were still present in the cecum and sigmoid colon. IFX (5 mg/kg) was then administered to induce remission. Five injections of IFX, which was tolerable without adverse events, led to complete remission in this case, as indicated by the complete disappearance of colonic ulcers through colonoscopy, and by improvement of the patient's symptoms (**Figure 2B**, bottom panel). Thus, this case of intestinal BD was successfully treated with IFX.

QUANTITATIVE PCR ANALYSES

Total mRNA was isolated from colonic biopsy samples during colonoscopy and subjected to quantitative PCR (qPCR) analyses as previously described (10). mRNA was isolated from colonic biopsy samples by using Monarch Total RNA Miniprep kit (New England Biolabs) and then was reverse transcribed into cDNA by High-Capacity RNA to cDNA kit (Applied Biosystems). The mRNA level for each target gene was determined by SYBR Green-based qPCR using a Step One Plus system (Applied Biosystems) and Quantitect primer assays (Qiagen) followed by the normalization using β-actin as a reference gene. Each PCR primer for Quantitect primer assays was purchased from Qiagen as follows; β-actin (#QT00095431), TNF-α (#QT00029162), IFN-β (#QT000203763), IFN-γ (#QT00000525), IL-6 (#QT00083720), IL-9 (#QT00000630), IL-12 (#QT00000364), IL-13 (#QT00000511), IL-33 (#QT00041559), C-C chemokine ligand 17 (CCL17, #QT00096866), CCL26 (#QT00023135), FOXP3 (#QT00048286), C-X-C motif chemokine ligand 9 (CXCL9, #QT00013461), and CXCL10 (#QT01003065). In some experiments, custom made primers were obtained from Invitrogen. Primer sequences were as follows; IL-1β (sense; GGACAAGCTGAGGAAGATGC, antisense; TCGTTATCCC

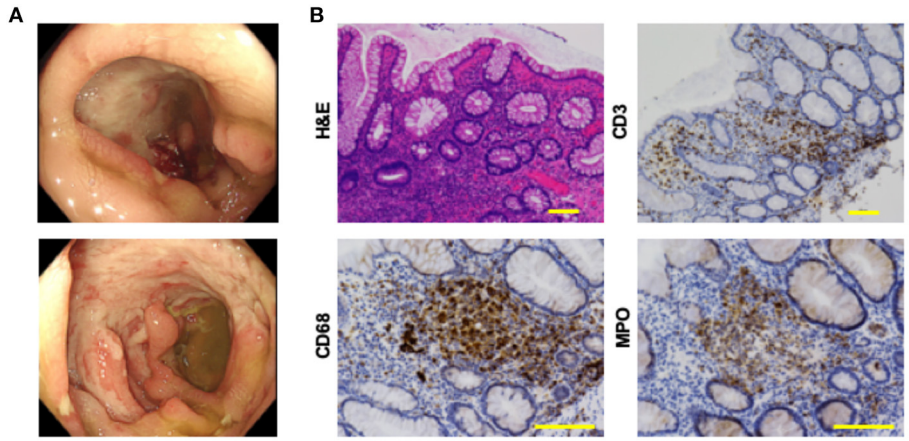

FIGURE 1 | Colonoscopic and pathological findings in a patient with intestinal Behçet's disease before the treatment. **(A)** Colonoscopy revealed punched-out, deep and semi-circumferential ulcers in the cecum (top), and sigmoid colon (bottom), respectively. **(B)** Colonic biopsy samples were subjected to hematoxylin and eosin (H&E) staining and immunohistochemical analysis to visualize $CD3^+$ T cells, $CD68^+$ macrophages, and myeloperoxidase $(MPO)^+$ granulocytes. Accumulation of immune cells and destruction of crypt architecture were observed in tissues stained by H&E. Colonic mucosa of this patient with intestinal Behçet's disease was characterized by the infiltration of $CD3^+$ T cells, $CD68^+$ macrophages, and MPO^+ granulocytes. Scale bar, 100 μm.

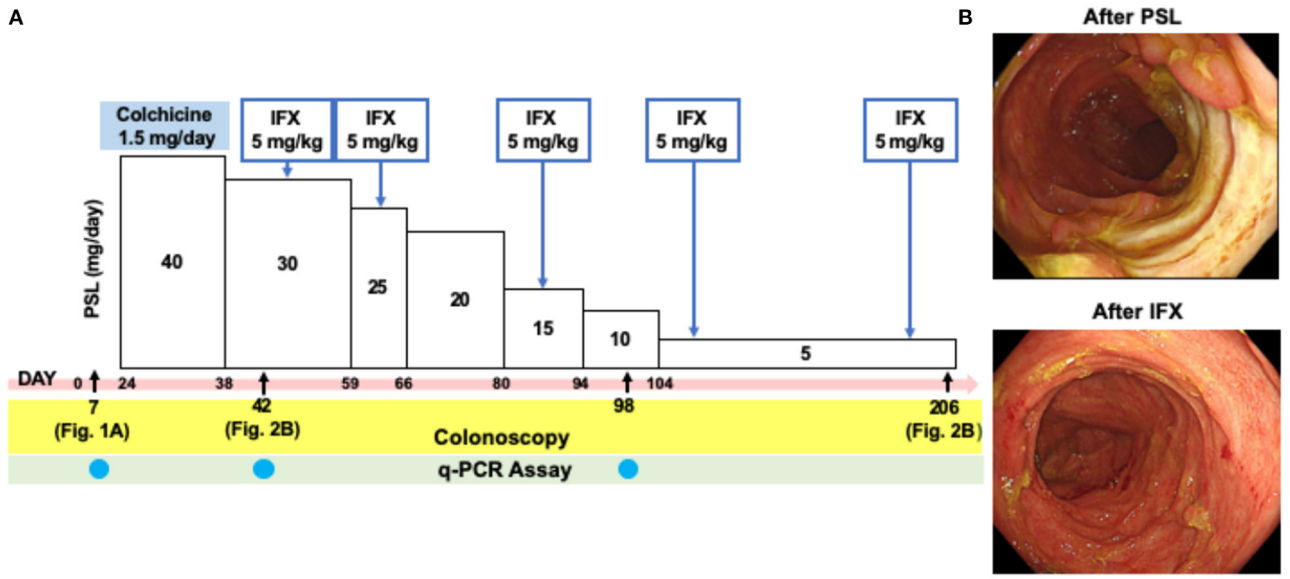

FIGURE 2 | Colonoscopic findings in a patient with intestinal Behçet's disease after the treatment with prednisolone and infliximab. **(A)** Clinical course of a patient with intestinal Behçet's disease. Day 0 was defined as on day of admission. **(B)** Colonoscopy performed after the treatment with prednisolone (PSL, Day 42) revealed semi-circumferential ulcers in the sigmoid colon. Disappearance of multiple ulcers was seen in the sigmoid colon after five times injections of infliximab (IFX, Day 206).

ATGTGTCGAA), IL-5 (sense; TGAGGATGCTTCTGCA TTTG, antisense; GCAGTGCCAAGGTCTCTTTC), IL-17 (sense; CATGAACTCTGTCCCCATCC, antisense; CCCAC GGACACCAGTATCTT), IL-23 (sense; TCTCCTTCTCCGCTT CAAAA, antisense; TTAGGGACTCAGGGTTGCTG). Quality of RNA and cDNA was checked by NanoDrop (Thermo Fisher Scientific). Annealing temperature was optimized and the specificity of the reaction was confirmed by the presence of a sharp single peak in melting curve analyses. mRNA expression levels of target genes were normalized by that of β-actin mRNA by using Ct method. Thus, qPCR analyses were performed in accordance with the MIQE guidelines.

The levels of mRNAs encoding proinflammatory cytokines and chemokines were compared in samples isolated before treatment (Day 7), after treatment with PSL (Day 42), and after three injections of IFX (Day 98). qPCR analyses revealed that the colonic mucosa before treatment was characterized by elevated mRNA expression levels of IL-17, IL-23, CXCL9,

CXCL10, and IFN-β (**Figure 3A**). Although, treatment with PSL led to a marked decrease in all proinflammatory cytokines and chemokines at mRNA level, remission was not achieved. Notably, successful induction of remission by IFX was accompanied by elevated mRNA expression levels of CXCL9, CXCL10, IFN-β, IFN-γ, IL-1β, IL-6, and IL-33 as compared with those after PSL treatment. IL-6 mRNA expression was particularly strongly induced by IFX, suggesting that IL-6 may be involved in the improvement of chronic inflammation in this case. Expression of colitogenic pro-inflammatory cytokines at mRNA level, such as IL-17, IL-23, and TNF-α were suppressed by both PSL and IFX treatments, compared to their levels before treatment. We also examined the mRNA expression of FOXP3 and found that treatment with IFX markedly increased its levels in the colonic mucosa (**Figure 3B**). Thus, increased expression of IL-6 and FOXP3 and decreased expression of TNF-α at mRNA level might be key factors in the resolution of severe inflammation in this case of intestinal BD.

DISCUSSION

Here, we report a case of intestinal BD that was successfully treated with IFX. This case was diagnosed as intestinal BD according to the international diagnostic criteria for BD (2). Our qPCR analyses of colonic biopsy samples revealed that the colonic mucosa of this patient was characterized by increased expression levels of IL-17, IL-23, TNF-α, IFN-β, CXCL9, and CXCL10 before treatment. Treatment with PSL decreased cytokine and chemokine expression levels in our qPCR analyses, which, however, did not result in the induction of remission. Interestingly, treatment switch from PSL to IFX led to complete remission of intestinal BD. The induction of complete remission by IFX was likely related to the marked increase in the expression levels of IL-6 and FOXP3 mRNA caused by this biologic drug, which was concomitant with the suppressed expression of IL-17, IL-23, and TNF-α mRNA.

Given that activation of IL-6-mediated signaling pathways underlies the immunopathogenesis of various autoimmune diseases, the increase in IL-6 expression by IFX was paradoxical (11). Indeed, serum concentrations of IL-6 are higher in patients with BD than in healthy controls (5). In this regard, Rakoff-Nahoum et al. (12) showed that regeneration of the intestinal epithelium depends upon the activation of IL-6-mediated signaling pathways in an experimental model of colitis. In this case, regeneration of the intestinal epithelium was promoted soon after treatment with IFX. Therefore, we hypothesize that the induction of IL-6 expression contributed to the remission of BD by promoting epithelial regeneration. However, we need to have a thorough understanding regarding the exact role played by IL-6 in BD, because tocilizumab, an anti-IL-6 receptor Ab, is effective in a significant fraction of patients with refractory uveitis associated with BD (13). Further, studies addressing molecular mechanisms accounting for the link between IL-6 and remission are therefore required to determine the beneficial roles of IL-6 in patients with BD.

Another important feature observed in our qPCR analyses was the increased expression of FOXP3, a critical transcription factor in Tregs (14). Activation of $FOXP3^+$ Tregs suppresses chronic inflammatory reactions (14). IFX treatment was accompanied by elevated FOXP3 expression in the colonic mucosa. Given the fact that Tregs expressing FOXP3 suppress colonic inflammatory responses, it is likely that induction of remission was partially achieved by the accumulation of Tregs in this case of intestinal BD (14). In agreement with this notion, neutralization of TNF-α by IFX or adalimumab led to increased expression of FOXP3 and accumulation of Tregs (7, 8). In this regards, augmented IL-33 responses induced by IFX treatment might be also involved in the FOXP3 expression since this cytokine has the ability to promote differentiation of Tregs in the gastrointestinal tract (15). All-trans retinoic acid, a metabolite of Vitamin A, induces differentiation of Tregs in the gastrointestinal tract (16). Therefore, Vitamin A in the diet might promote Treg differentiation. However, the relationship between Vitamin A uptake and BD pathogenesis has been poorly defined.

In contrast to the results of the IFX treatment, the treatment with PSL markedly reduced expression levels of all cytokines and chemokines in our qPCR analyses. Furthermore, PSL treatment decreased FOXP3 mRNA expression, indicating that PSL suppressed a broad range of immune responses. Consistent with these data, Ogawa et al. (17) provided evidence that PSL inhibits nuclear translocation of nuclear factor κB and interferon regulatory factors, thereby suppressing the production of cytokines and chemokines. In addition to the suppression of typical colitogenic cytokines such as IL-17, IL-23, and TNF-α (18), treatment with PSL notably diminished type I IFN responses (IFN-β, CXCL9, and CXCL10). Given that type I IFN cytokines and chemokines constitute a part of mucosal host defense against gut microorganisms, the failure of PSL to induce remission might be partially explained by the impaired mucosal host defenses due to the impaired activation of type I IFN responses (19). Thus, mechanisms of action differ between PSL and IFX, in that the latter promotes the expression of IL-6 and FOXP3, whereas, the former suppresses almost all immune responses. Such differences likely explain the observation that IFX was able to successfully induce remission, whereas, PSL lacked this therapeutic ability.

Treatment with IFX led to enhanced expression of IL-6 and FOXP3 at mRNA level, but not protein level. Thus, we have not confirmed expression of IL-6 or FOXP3 at protein level. Given that IL-6 and FOXP3 are subjected to post-transcriptional and post-translational processes, it is possible that increased expression of IL-6 and FOXP3 at mRNA level does not lead to functional expression at protein level. We have to be cautious regarding the interpretation of increased expression of IL-6 and FOXP3 at mRNA level. Therefore, further studies addressing expression of IL-6 and FOXP3 at protein level are absolutely required to verify our idea that enhanced expression of IL-6 and FOXP3 is associated with the induction of remission of intestinal BD by IFX. In addition, we cannot completely exclude a possibility that cytokines other than IL-6, e.g., TGF-β1 and IL-10, might play beneficial roles in the resolution of chronic intestinal inflammation in this case (14, 16).

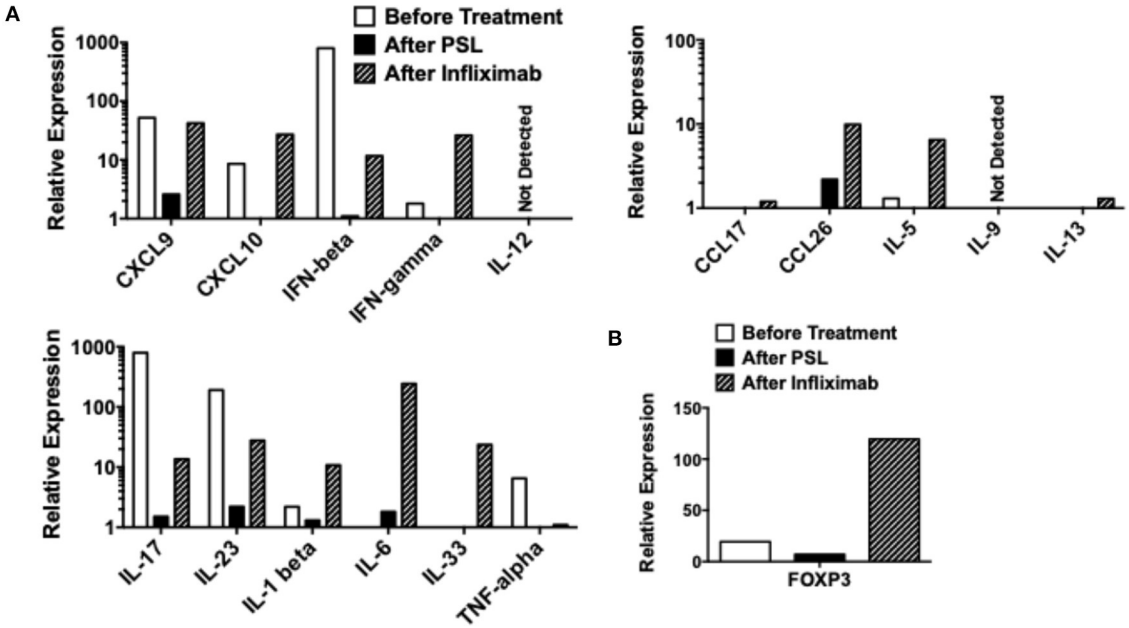

FIGURE 3 | Quantitative polymerase chain reaction analyses in colonic biopsy samples. Colonic biopsy samples were subjected to quantitative polymerase chain reaction analyses to determine expression of cytokines and chemokines. Colonic biopsy samples were obtained before the treatment (Day 7 in **Figure 2A**), after the treatment with prednisolone (PSL, Day 42 in **Figure 2A**), and after the treatment with infliximab (IFX, Day 98 in **Figure 2A**). mRNA expression levels of cytokines, chemokines **(A)**, and forkhead box P3 (FOXP3) **(B)** were normalized by that of β-actin mRNA.

In conclusion, we described a case of intestinal BD that was successfully treated with IFX. Our extensive qPCR analyses of colonic biopsy samples before and after treatment with PSL or IFX revealed alterations in the expression levels of cytokines and chemokines. Increased mRNA expression of IL-6 and FOXP3 was associated with the remission induced by IFX in this case with intestinal BD. Thus, treatment with IFX has dual roles in the suppression of chronic inflammation in BD. First, IFX treatment might promote regeneration of the epithelium through the induction of IL-6 expression (12). Second, IFX treatment might suppress inflammation through the activation of $FOXP3^+$ Tregs (7, 8). Alterations in IL-6 and FOXP3 mRNA expression levels seen in this case partially explain the molecular mechanisms by which IFX suppresses autoimmune reactions in intestinal BD. Future, studies utilizing a large number of patients with intestinal BD are required to verify this hypothesis.

DATA AVAILABILITY STATEMENT

The datasets presented in this article are not readily available because this is a case report and a dataset has not been generated. Requests to access the datasets should be directed to tomohiro@med.kindai.ac.jp.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Kindai University Faculty of Medicine. The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the patient for the publication of this report.

AUTHOR CONTRIBUTIONS

KY, IS, RT, AH, MKur, YM, YO, SM, and YK took care of the patient. KY and TW drafted the manuscript and performed the experiments. TY, KK, KM, and MKud edited and revised the manuscript. TC performed pathological analyses. 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/fmed.2021.679237/full#supplementary-material

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HMGB1 Activates Myeloid Dendritic Cells by Up-Regulating mTOR Pathway in Systemic Lupus Erythematosus

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Research has shown that HMGB1 can activate dendritic cells (DCs), but its molecular mechanisms are not clear. In this study, we reported that the myeloid dendritic cells (mDCs) were activated in the peripheral blood of SLE patients, and the activation of mDCs was associated with the up-regulation of HMGB1 and mTOR. After stimulated by HMGB1, expression of mTOR and its substrates P70S6K and 4EBP1 in dendritic cells increased considerably ($P < 0.01$). The expression of HLA-DR, CD40, and CD86 on dendritic cells also significantly increased following these stimuli ($P < 0.01$). In addition, stimulation with HMGB1 enhanced cytokine (IL-1β, IL-6, and TNF-a) production in dendritic cells. In contrast, the HMGB1-mediated expression of HLA-DR, CD40, and CD86 on dendritic cells and production of IL-1β, IL-6, and TNF-α were reduced by rapamycin. Rapamycin can inhibit HMGB1-induced activation of mDCs and secretion of pro-inflammatory cytokines. These findings indicated that HMGB1activates mDCs by up-regulating the mTOR pathway in SLE.

Keywords: dendritic cells, HMGB1, signal transduction pathway, systemic lupus erythematosus, mTOR inhibitor

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INTRODUCTION

Systemic lupus erythematosus (SLE) is a severe, debilitating autoimmune disease that affects multiple organs and body systems. The prevalence of SLE worldwide is estimated to be as high as 150 per 100,000 individuals (1). The disease is characterized by autoantibodies against nuclear antigens (ANA), which result from immune system deregulation (2). Although research has been done, the pathogenesis of SLE is not yet fully understood.

Dendritic cells (DCs) play an essential role in bridging the innate and adaptive immune systems. DCs are antigen-presenting cells displaying the unique capability to activate naïve T cells. DCs can also respond to encounter pathogens by producing inflammatory mediators, including proinflammatory cytokines (3). Because of these complex roles, an imbalance in DC functions can cause a defective or exaggerated immune response and tissue damage. Research has shown that HMGB1, a non-histone nuclear protein, can induce immune responses and inflammatory responses that are relevant for the pathogenesis of SLE (4). For example, recent evidence indicates that HMGB1 is responsible for producing proinflammatory cytokines, which is a well-established damage associated molecular pattern (DAMP) (5–7). HMGB1 is likely to be released from

activated immune cells such as dendritic cells (DCs) in inflammation or injury (8). When released, HMGB1 participates in the secretion of downstream proinflammatory cytokines *via* binding to cell surface receptors such as receptor of advanced glycation end products (RAGE), TLR2, and TLR4, contributing to the occurrence and development of diverse inflammatory diseases and autoimmune diseases (9–14).

One signaling pathway that has been the subject of current research in SLE is the mechanistic target of rapamycin (mTOR). Recent studies have found that activation of mTOR in both the immune system (15, 16) and non-traditional parenchymal organs (e.g., the liver) precedes the onset of disease and represents early manifestations of pathogenesis (17). A common finding in T cells, B cells, macrophages, hepatocytes, and renovascular cells is mTOR activation (18). But little research has been done on mTOR activation in dendritic cells in SLE. Therefore, this study aimed to assess the potential role of mTOR in the activation of myeloid dendritic cells (mDCs) triggered by HMGB1 in patients with SLE.

MATERIALS AND METHODS

Design

The study used a case-control design to examine whether HMGB1 can contribute to the up-regulation of the mTOR pathway of dendritic cells in patients with SLE. We collected peripheral blood samples from patients with SLE and healthy controls (HCs). We quantified the level of HMGB1 with ELISA and analyzed mDCs from peripheral blood with flow cytometry. Next, we stimulated mDCs from SLE with HMGB1. The expression of mTOR and its substrates, HLA-DR, costimulatory molecules of dendritic cells, and cytokine synthesis were measured. These indexes were measured again after blocking the mTOR pathway with rapamycin.

Participants

We recruited 35 patients with SLE, who fulfilled the American College of Rheumatology (ACR) revised criteria for the classification of SLE (19), and 20 healthy controls. SLE disease activity index 2000 (SLEDAI-2K) (20) was determined in the blood sampling. Patients with SLE were recruited from the Department of Rheumatism and Immunology, the First Affiliated Hospital of Guangxi Medical University. All patients were newly diagnosed and did not receive any corticosteroids or immunosuppressive treatment at the time of the blood collection. Healthy adults matched for age and sex were enrolled from blood donors. The study was approved by the Medical Ethical Committee of the First Affiliated Hospital of Guangxi Medical University (NO.2017-KY-国基-111) and the Medical Ethical Committee of the Fourth Affiliated Hospital of Guangxi Medical University (NO. KY2018083), China. All subjects signed the informed consent.

Abbreviations: DCs, dendritic cells; SLE, Systemic lupus erythematosus; mDCs, myeloid dendritic cells; HMGB1, high-mobility group box protein-1; mTOR, mechanistic target of rapamycin; HLA-DR, human leukocyte antigen DR; DAMP, damage associated molecular pattern; RAGE, receptor of advanced glycation end products; ACR, American College of Rheumatology; PBMC, Peripheral blood mononuclear cells; Rapa, rapamycin; HCs, healthy controls.

Quantitation of HMGB1

HMGB1 in serum samples of 35 SLE patients and 20 healthy controls were analyzed with ELISA kits (CUSABIO) according to the manufactural protocol. Plates were read at an absorbance of 450 nm (A450) using a Sunrise microplate reader. All measurements were carried out in duplicate.

Analysis mDCs From Peripheral Blood of SLE With Flow Cytometry

Peripheral blood mononuclear cells (PBMC) were isolated from the peripheral blood samples of SLE patients by using Lymphoprep^{TM} (STEMCELL). Freshly isolated PBMCs were stained for surface marker FITC anti-human Lineage Cocktail (CD3, CD14, CD19, CD20, CD56) (BD Pharmingen), PerCp anti-human HLA-DR (BD Pharmingen), PE anti-human CD11c (BD Pharmingen), APC anti-human CD40 (BD Pharmingen) or APC anti-human CD86 (BD Pharmingen). After surface staining, cells were fixed/permeabilized with fixation/permeabilization solution (Cytofix/Cytoperm^{TM}; BD Pharmingen), and stained with p-mTOR-Alexa 647 (BD Pharmingen) for 30 min at 4°C. Cells were then washed with 1 × Perm/Wash Buffer (BD Pharmingen) and resuspended in PBS + 2% FBS for flow cytometric analysis. Flow cytometry was performed on a BD FACS Canto II (BD Biosciences) and data were analyzed using FCS Express 4 software (De Novo Software, Los Angeles, CA).

Preparation and Stimulation of mDCs

Whole blood samples from 20 patients with SLE were collected using ACD tubes (BD Vacutainer). Peripheral blood mononuclear cells (PBMCs) were isolated using Lymphoprep^{TM} (STEMCELL). Cells were cultured in RPMI-1640 supplemented with 10% FBS at a density of 2×10^6 cells/ml in 6-well tissue culture plates. After PBMC were cultured for 3 h, the liquid in the containers was discarded. Then RPMI-1640 was added for further culture, supplemented with 10% FBS, recombinant human GM-CSF (100 ng/ml; PeproTech), and recombinant human IL-4 (100 ng/ml; PeproTech). On days 2 and 4 of culture, the supernatant was removed and replaced with fresh medium containing hGM-CSF and hIL-4. All cultures were incubated at 37°C in 5% humidified CO_2. After seven days of culture, more than 95% of the cells expressed CD11c +, the characteristic DC-specific markers, as determined by FACS (**Supplementary Figure 1**).

To determine the effect of HMGB1 on mDC activation, primary mDCs were stimulated with HMGB1 (1 ug/ml; Peprotech) on Day 6 of culture. Cells were then collected after 24 h.

To block the mTOR pathway, we added different concentrations of rapamycin (10, 20, and 40 ng/ml) to primary mDCs on Day 5 of culture, and then stimulated with HMGB1 as described above.

Measurement of Cytokine Production and Surface Molecules of mDCs

Levels of various cytokines (IL-1β, IL-6, and TNF-α) in the supernatant of mDCs cultures were quantified using ELISA analysis (BD Biosciences).

The surface molecules on dendritic cells were measured by flow cytometry. Cultured mDCs were stained for surface markers PerCp anti-human HLA-DR, PE anti-human CD11c, APC anti-human CD40, or APC anti-human CD86. All antibodies and isotype control were purchased from BD Pharmingen. Cell surface staining was performed according to the standard procedures.

The Expression of mTOR in mDCs by RT-PCR

Total cellular RNAs were isolated from DCs using AxyPrep Multisource Total RNA Miniprep Kit (Axygen), and RNA samples were transformed into complementary DNA (cDNA) using RevertAid First-strand cDNA Synthesis kit (Thermo) according to the manufacturer's instructions. Quantitative PCR was performed on selected targets (mTOR, P70S6K, and 4EBP1) using pre-developed primers and probes (Sangon Biotech) on the QuantStudio (TM) real-time PCR software System. β-actin was used as an internal control. Primer sequences were the following: β-ACTIN, 5′-CCT GGC ACC CAG CAC AAT-3′ and 5′ -GGG CCG GAC TCG TCA TAC-3′; mTOR, 5′-ACT GGA GGC TGA TGG ACA CA-3′ and 5′-GGC TCT CCA AGT TCC ACA CC-3′; P70S6K, 5′-CAT CGG CAC CAC TTC CAA TA-3′ and 5′-TTC ATA CGC AGG TGC TCT GG-3′; 4EBP1, 5′-TCG GAA CTC ACC TGT GAC CA-3′ and 5′-GCT CAT CAC TGG AAG GGC TG-3′. Expression of mTOR, P70S6K, and 4EBP1 were calculated as described by the manufacturer.

Statistical Analysis

Graph Pad Prism version 7 (GraphPad Software Inc., San Diego, CA, USA) was used to perform comparisons between different groups and to generate figures. Calculations were based on a 95% confidence interval (CI). *P*-values <0.05 were considered significant. All data were expressed as the mean ± SD. Student's *t*-test was used for continuous variables that were normally distributed. The Mann–Whitney *U*-test was used for continuous variables that were not normally distributed.

RESULT

We included 35 subjects with SLE and 20 healthy controls. All patients were newly diagnosed with SLE and did not receive any corticosteroids or immunosuppressive treatment at the time of the blood draw. Most patients with SLE were women (29/35, 83%), with a mean age of 40 ± 13.27 years. They had a SLEDAI score of 9.9 ± 4. Common disease activity at the time of inclusion was renal (66%). Healthy controls (HCs) consisted of 20 healthy individuals matched for sex and age. Other characteristics of the patients are summarized in **Supplementary Table 1**.

mDCs Were Activated in the Peripheral Blood of SLE Patients, and the mDCs Activation Was Associated With the Up-Regulation of HMGB1 and mTOR

Compared to healthy controls (HCs), mDC in the peripheral blood of SLE patients expressed more CD40 and CD86 (**Figures 1A–C**). The levels of CD40 and CD86 were positively correlated with levels of HMGB1 and p-mTOR in mDCs (**Figure 1D**).

Mean serum level of HMGB1 in SLE patients was significantly higher than that in healthy controls (HCs) (66.570 ± 17.995 pg/ml vs. 53.265 ± 8.727 pg/ml, $p < 0.001$). HMGB1 levels were positively correlated with SLEDAI scores ($r = 0.817$, $p < 0.001$, **Figure 2E**).

The expression of p-mTOR in mDCs increased significantly in SLE patients (**Figures 2A–D**). The levels of p-mTOR in mDCs were positively correlated with SLEDAI scores ($r = 0.943$, $p < 0.001$, **Figure 2F**) and the levels of HMGB1 in peripheral blood ($r = 0.805$, $p < 0.001$, **Figure 2G**), respectively.

HMGB1 Activated Myeloid Dendritic Cells and Up-Regulated mTOR Pathways in mDCs From SLE Patients

The mDCs from 20 SLE patients were isolated and induced to mature with cytokines, then stimulated with HMGB1. Compared to the healthy control group, mDCs stimulated by HMGB1 expressed more HLA-DR, CD40, and CD86 in SLE patients (**Figure 3**) and produced significantly more TNF-α, IL-6, and IL-1β (**Figure 4**). Moreover, HMGB1 induced up-regulation of mTOR and its substrates (P70S6K, 4EBP1) in mDCs (**Figure 5**). Therefore, HMGB1 can induce activation of mDCs from patients with SLE and up-regulate mTOR pathway in mDCs.

Activation of mDCs Could Be Inhibited by Blocking the mTOR Pathway With Rapamycin

We inhibited the mTOR pathway with different concentrations of rapamycin (Rapa). The mRNA expression of mTOR and its substrate P70S6K and 4EBP1 in mDCs was considerably decreased through Rapa intervention by RT-PCR (**Figure 6** and **Supplementary Table 2**). These results suggested that rapamycin can inhibit the activation of the mTOR pathway in mDCs induced by HMGB1. Furthermore, rapamycin can inhibit increased expression of HLA-DR, CD40, CD86 (**Figure 7** and **Supplementary Table 3**) and increased secretion of TNF-α, IL-6, and IL-1 induced by HMGB1 (**Figure 8** and **Supplementary Table 4**), respectively. Overall, these results suggested that HMGB1-induced activation of mDCs could be affected by blocking the mTOR pathway with rapamycin.

DISCUSSION

SLE, a systemic autoimmune disease, is a potentially fatal disease characterized by immune complex deposition and the subsequent inflammation that contribute to severe tissue damage (21). Recent reports show that HMGB1 might be involved in autoimmune and inflammatory diseases, including SLE (22–24). Previous studies and our results revealed that the level of HMGB1 was positively correlated with SLEDAI score in SLE patients, implying that the critical role of HMGB1 in the pathogenesis of SLE.

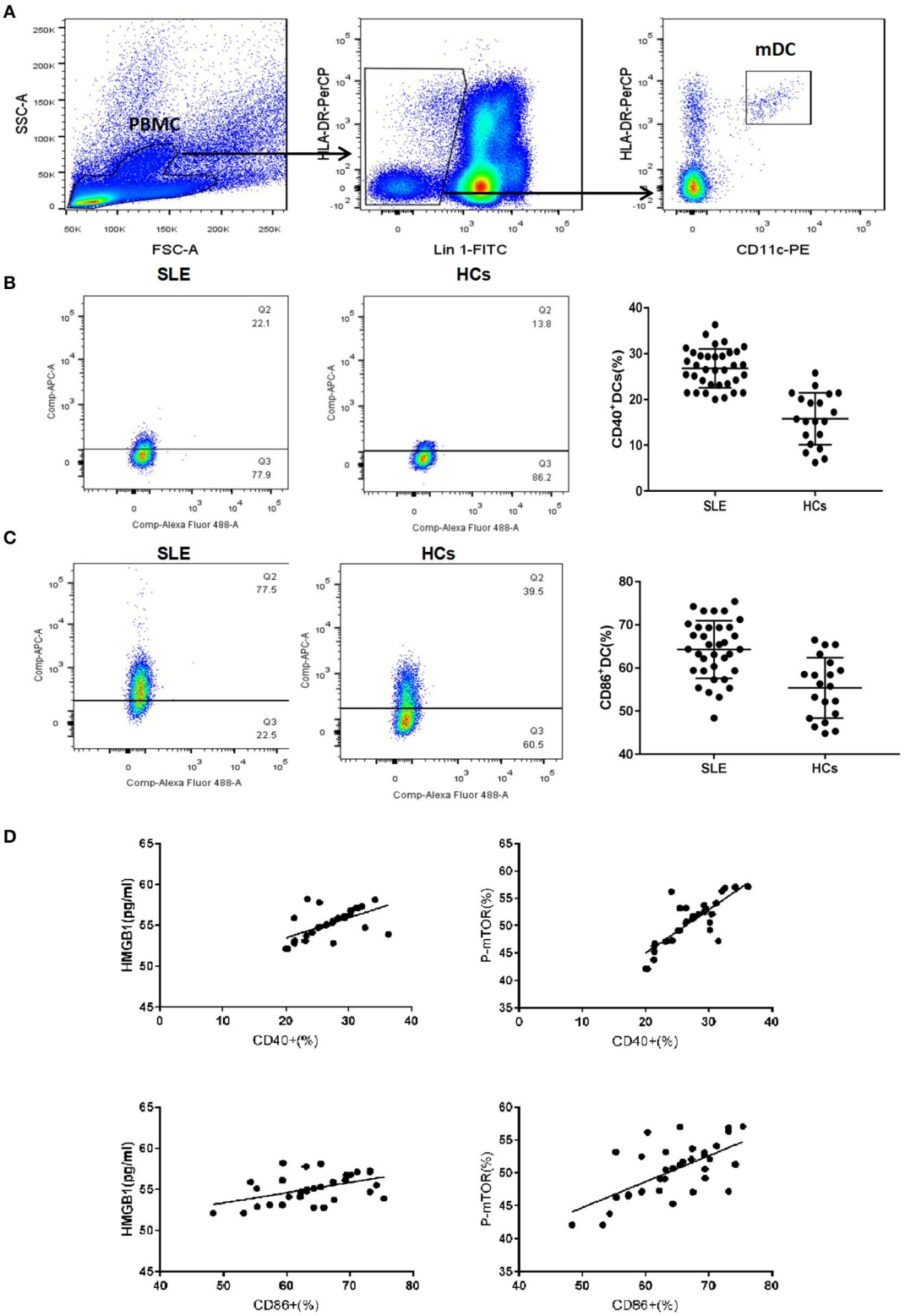

FIGURE 1 | mDCs phenotype of SLE and healthy control group. **(A)** Gating strategy for flow cytometry analysis of mDCs. **(B)** The mDC in peripheral blood of SLE patients to express more CD40 than HCs (26.79 ± 4.23% vs. 15.78 ± 5.68%, $t = 8.185$, $p = 0.000$). **(C)** The mDC in peripheral blood of SLE patients to express more CD86 than HCs (64.27 ± 6.68% vs. 55.38 ± 7.01%, $t = 4.664$, $p = 0.000$). **(D)** The levels of CD40 and CD86 were positively correlated with the levels of HMGB1 (CD40: $r = 0.600$, $P = 0.001$; CD86: $r = 0.478$, $P = 0.004$) and p-mTOR in mDCs (CD40: $r = 0.812$, $P = 0.001$; CD86: $r = 0.640$, $P = 0.001$).

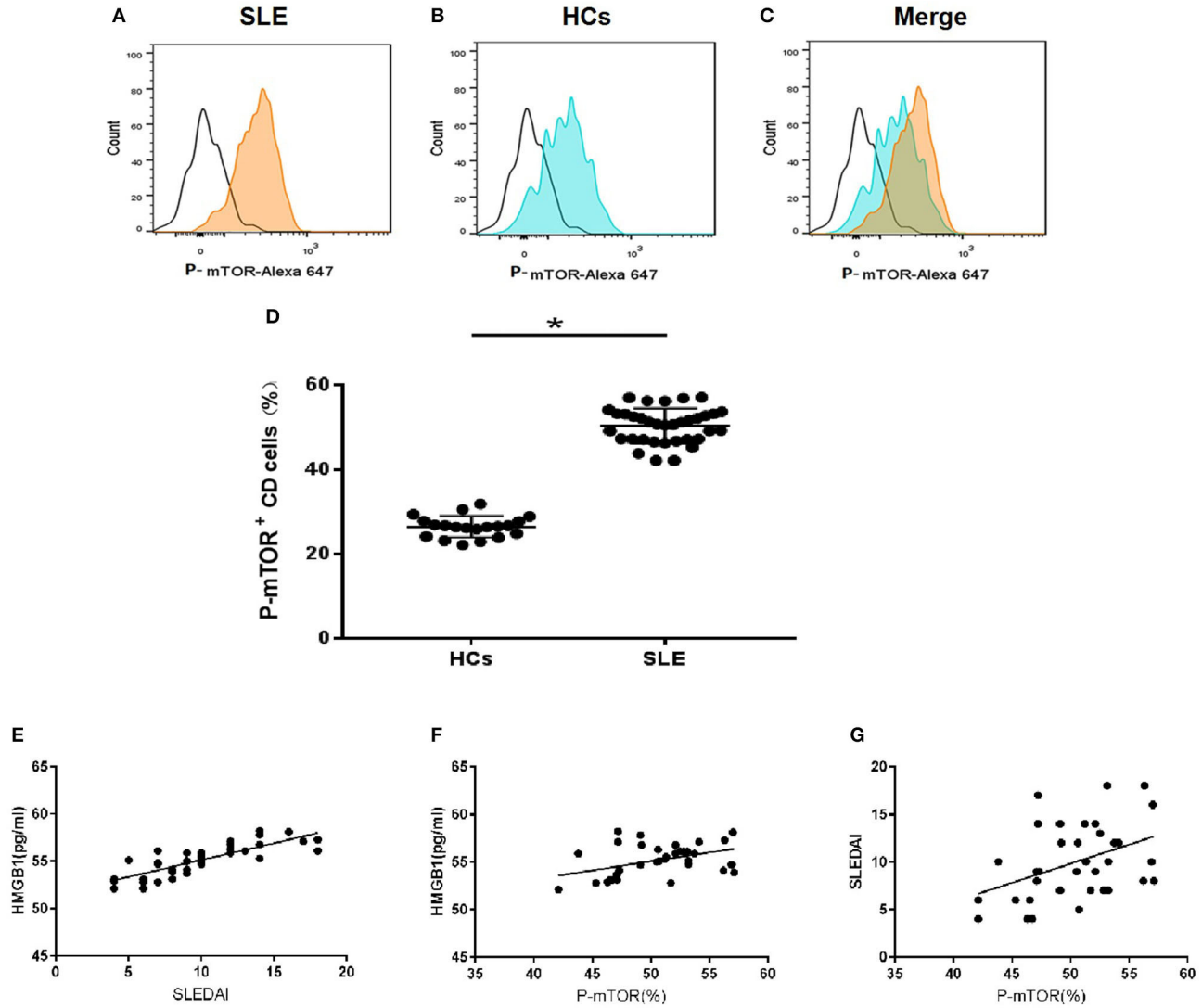

FIGURE 2 | HMGB1 was unregulated in SLE patients and positively correlated with levels of p-mTOR in mDCs. **(A)** The level of p-mTOR in DCs from SLE patients; **(B)** The level of p-mTOR in DCs from healthy controls (HCs); **(C)** The merged figure of A with B; **(D)** The level of p-mTOR in DCs from SLE patients was higher than HCs (50.35 ± 0.696% vs. 26.43 ± 0.565%, $P = 0.027$); *$P < 0.05$. **(E)** The correlation between serum HMGB1 concentrations and SLE disease activity index score (SLEDAI) in SLE patients. In peripheral blood, HMGB1 levels were positively correlated with SLEDAI scores ($r = 0.817$, $p < 0.001$). **(F)** The correlation between the SLEDAI and p-mTOR in mDCs in peripheral blood of SLE patients. Levels of p-mTOR in mDCs were positively correlated with the SLEDAI ($r = 0.417$, $p < 0.0128$). **(G)** The correlation between the levels of HMGB1 and p-mTOR in mDCs in peripheral blood of SLE patients. Levels of p-mTOR in mDCs positively correlated with the levels of HMGB1 in peripheral blood ($r = 0.446$, $p < 0.0072$).

Recent studies indicated that HMGB1, a well-established DAMP, is responsible for triggering inflammatory responses (7). HMGB1 is likely to be released from activated immune cells such as macrophages in the area of inflammation or injury (12, 13). In this study, we found that mDCs were activated in SLE. The levels of CD40 and CD86 were positively correlated with SLEDAI scores, and the activation of mDCs was associated with up-regulation of HMGB1 and mTOR. The expression of mTOR in mDCs increased in SLE, and the levels of mTOR in mDCs were positively correlated with SLEDAI scores and the levels of HMGB1 in peripheral blood, respectively. These findings implied that HMGB1 might induce activation of mDCs in patients with SLE and up-regulation of the mTOR pathway.

In this study, the results indicated that HMGB1 can activate dendritic cells and induce more proinflammatory cytokines (TNF-α, IL-6, and IL-1β). These results were consistent with previous studies (25). Persistent elevation of proinflammatory cytokines could lead to immune deregulation followed by local inflammatory processes and tissue damage (26, 27). In another study (28), the surface level of CD86 on monocytes in SLE was comparable with that in HCs. CD163 is an anti-inflammatory marker, whereas HLA-DR is a proinflammatory marker. These

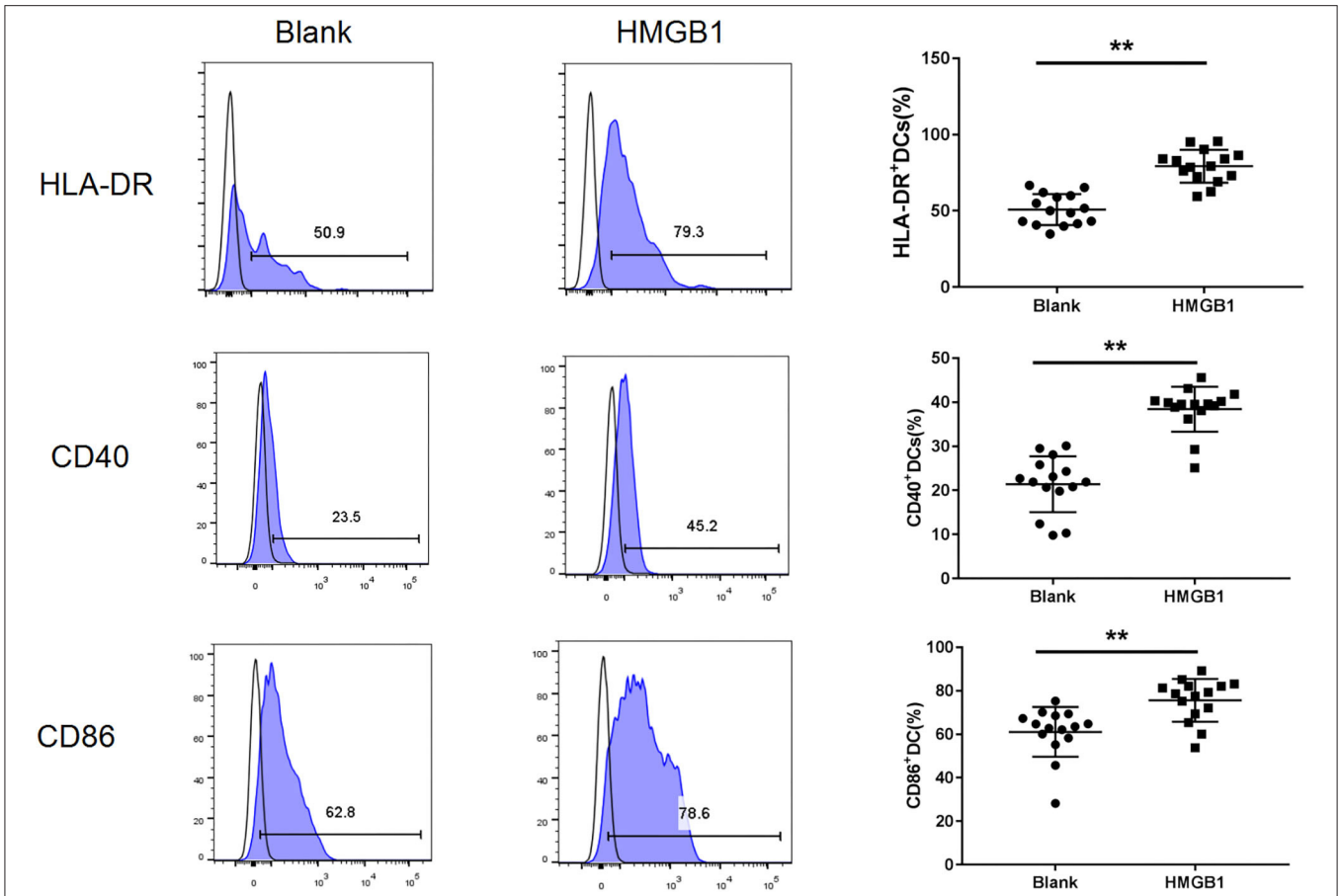

FIGURE 3 | HMGB1 promoted activation of mDCs from SLE patients. Flow cytometry analysis of HLA-DR, CD40, and CD86, surface expression on each group mDCs from SLE patients. The expression of HLA-DR (79.27 ± 10.78% vs. 50.77 ± 10.14%, $t = 7.455$, $P = 0.00$), CD40 (38.42 ± 5.10% vs. 21.41 ± 6.33%, $t = 8.102$, $P = 0.000$) and CD86 (75.63.42 ± 9.80% vs. 61.07 ± 11.48%, $t = 3.735$, $P = 0.001$) were up-regulated on mDCs after stimulation with HMGB1.**$P < 0.001$.

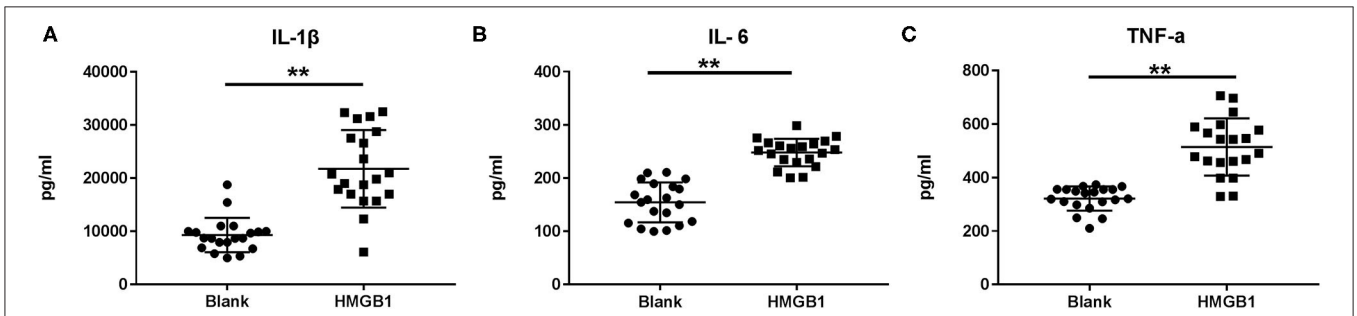

FIGURE 4 | Cytokine production following stimulation of HMGB1 measured by ELISA. When stimulated with HMGB1, the mDCs from systemic lupus erythematosus (SLE) patients produced significantly more IL-1β **(A)**, IL-6 **(B)**, and TNF-α **(C)** than the Blank. Data are presented as the median and interquartile range (IL-1β: 21745 ± 1632.45pg/ml vs. 9282.6 ± 723.68 pg/ml, $t = 6.979$, $P = 0.000$; IL-6: 248.01 ± 5.81 pg/ml vs. 154.41 ± 8.39 pg/ml, $t = 9.167$, $P = 0.000$; TNF-α: 514.41 ± 23.90 pg/ml vs. 321.78 ± 10.09 pg/ml, $t = 7.423$, $P = 0.000$) **$p < 0.001$. IL, interleukin; TNF, tumor necrosis factor.

data demonstrated the downregulation of proinflammatory surface markers but the upregulation of anti-inflammatory markers in SLE, which was different from the presumptive results. This discrepancy might be due to an existing negative feedback to maintain monocyte homeostasis in SLE.

mTOR plays a crucial role in the relationship among HMGB1, activation of mDCs, and autoimmunity in SLE. In this study, when stimulated by HMGB1, mDCs expressed increased mTOR and its substrates (P70S6K, 4EBP1) compared to the healthy control group. When the mTOR pathway

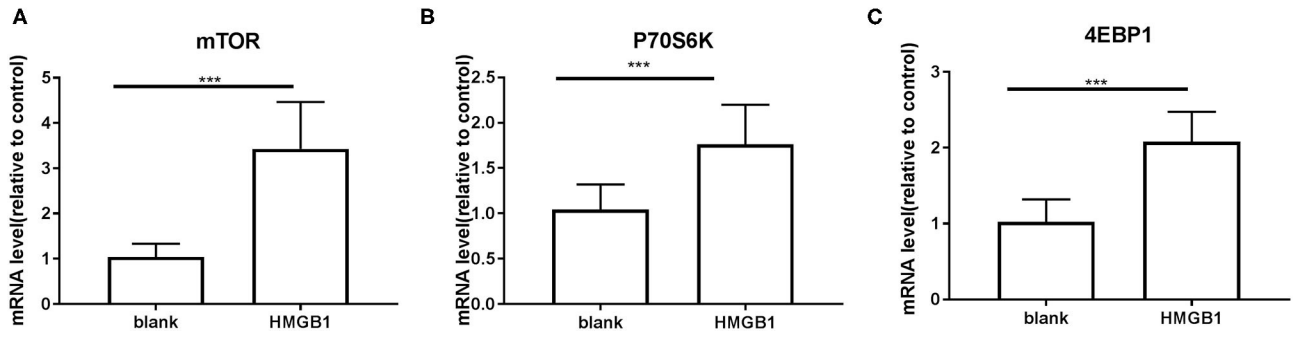

FIGURE 5 | mRNA expression of mTOR and its substrates P70S6K and 4EBP1 on mDCs measured by RT-PCR. Application of the $2^{-\Delta\Delta CT}$ method. The expression of mTOR **(A)**, P70S6K **(B)**, and 4EBP1 **(C)** were up-regulated in mDCs after stimulation with HMGB1. (mTOR: 1.039 ± 0.0651 vs. 3.428 ± 0.2315, $t = 9.935$, $P = 0.000$; P70S6K: 1.042 ± 0.06223 vs. 1.763 ± 0.09726, $t = 6.245$, $P = 0.000$ 4EBP1: 1.021 ± 0.066 vs. 2.078 ± 0.08861, $t = 9.546$, $P < 0.000$.) ***$P < 0.001$.

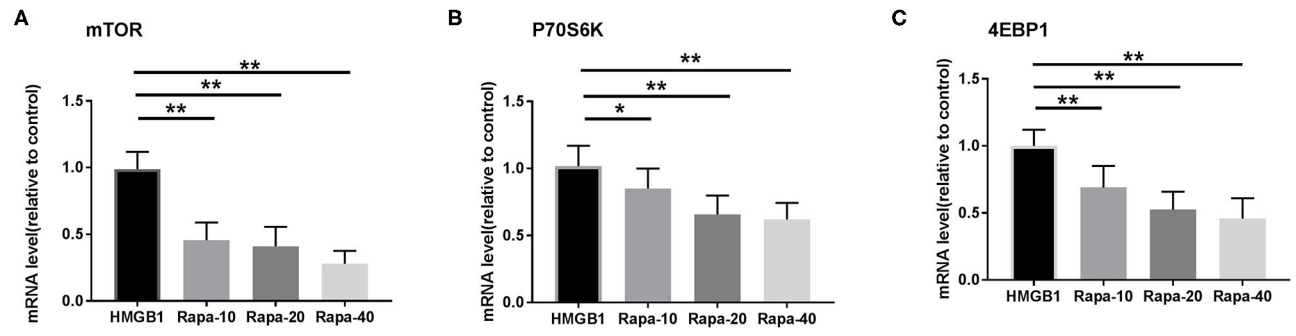

FIGURE 6 | mRNA expression of mTOR and its substrates P70S6K and 4EBP1 in mDCs under different concentrations of rapamycin (Rapa-10: 10 ng/ml; Rapa-20: 20 ng/ml; Rapa-40: 40 ng/ml) intervention by RT-PCR. Application of the $2^{-\Delta\Delta CT}$ method. The expression of mTOR **(A)**, P70S6K **(B)**, and 4EBP1 **(C)** were significantly decreased in mDCs through Rapa intervention. *$p < 0.05$, **$p < 0.01$.

was blocked with different concentrations of rapamycin (Rapa), HMGB1-induced mDC expression of HLA-DR, CD40, and CD86, and proinflammatory cytokines secretion was decreased. All these results implied that HMGB1 may induce activation of mDCs through the up-regulated mTOR pathway, but further research is needed to confirm this hypothesis.

It is well-known that mTOR signaling senses extracellular stimulations and regulates many biological processes including inflammation (29). Activation of mTOR delivers an obligatory signal for the proper activation and differentiation of mDCs in our present study. Therefore, the activation of mTOR signaling pathway is a potentially significant factor contributing to the pathogenesis of SLE. A recent study found that mTOR signaling activation caused hyperpolarization of mitochondria, resulting in necrosis tendency of T cells, promoting the generation of anti-nuclear antibodies, activation of dendritic cells, and the occurrence of inflammation (30).

In the present study, we intended to study upstream regulators of the mTOR pathway and its function in mDCs. Our data showed that rapamycin can inhibit HMGB1-induced activation of mDCs and secretion of pro-inflammatory cytokines. The next question would be to clarify the pathophysiological functions of mTOR in SLE and investigate targeted drugs. Given the general importance of the mTOR signaling pathway and considering the ubiquitous expression of HMGB1, the newly uncovered regulation is expected to have a broad impact. It includes an impact on metabolic programs and cell fate decisions in other immune and non-immune cells under homeostasis, when faced with an environmental challenge, and during the development of the autoimmune disease.

Therefore, blocking mTOR signaling pathway becomes a new target for the treatment of SLE. Rapamycin, a widely recognized blocker of the mTOR pathway, has a promising prospect in treating SLE. Rapamycin can not only inhibit the signal transduction downstream of mTOR, but also negatively regulate the PI3K/AKT /mTOR pathway. The monotherapy of rapamycin can completely prevent nephritis in mice and significantly improve the condition. Gu's study (31) demonstrated that RAPA alleviated the clinical symptoms of lupus nephritis and prolonged survival in MRL/lpr mice. This result is consistent with our previous research (32).

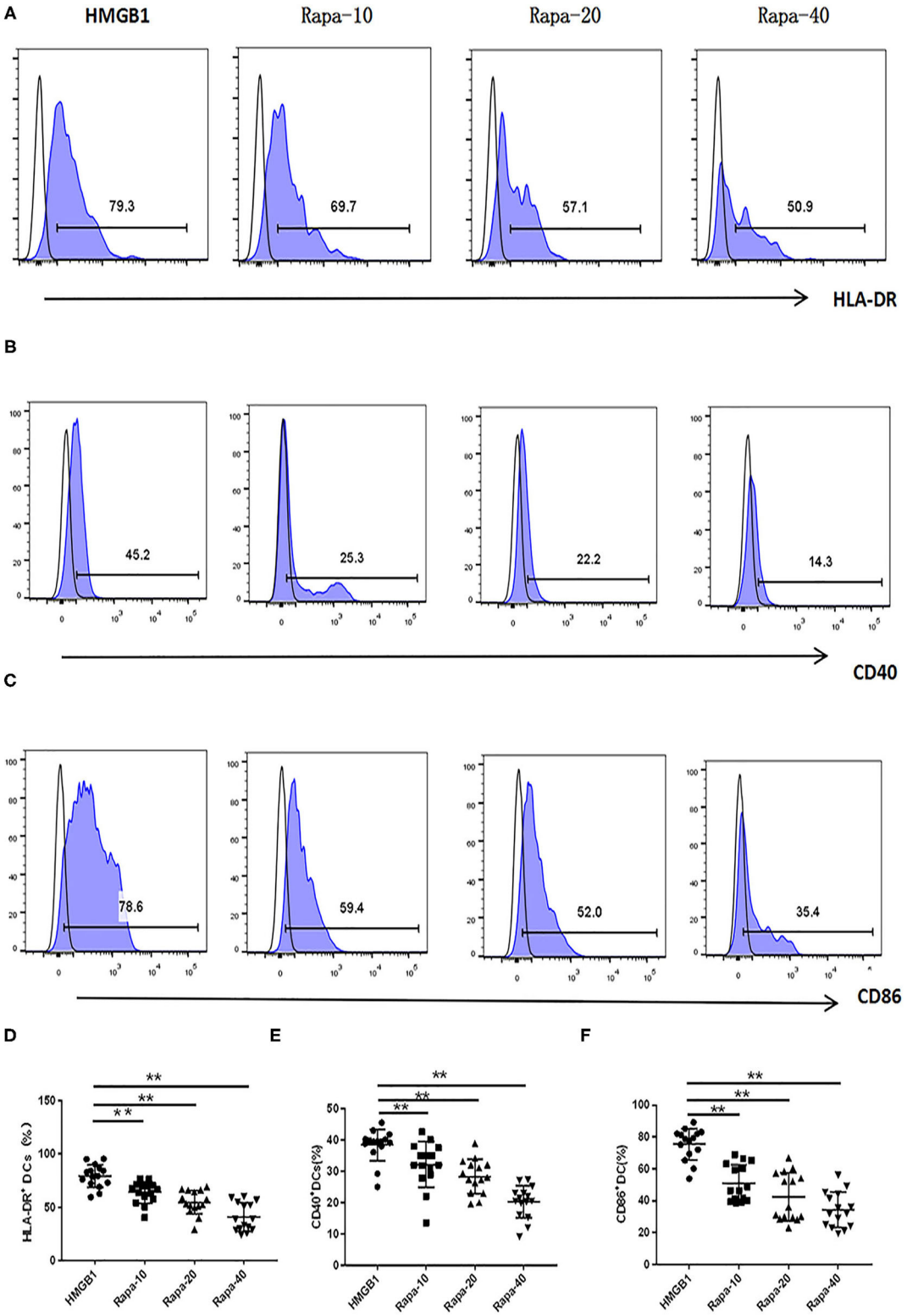

FIGURE 7 | Rapamycin can inhibit HMGB1-induced mDC expression of HLA-DR, CD40 and CD86 increase. Different concentrations of rapamycin (Rapa-10: 10 ng/ml; Rapa-20: 20 ng/ml; Rapa-40: 40 ng/ml) were put into cultures of primary mDCs. Flow cytometry analysis of HLA-DR **(A)**, CD40 **(B)** and CD86 **(C)**, surface expression on each group mDCs from SLE patients. The expression of HLA-DR **(D)**, CD40 **(E)**, and CD86 **(F)** were decreased on mDCs through Rapa intervention compared with HMGB1 group. $^{**}P < 0.01$.

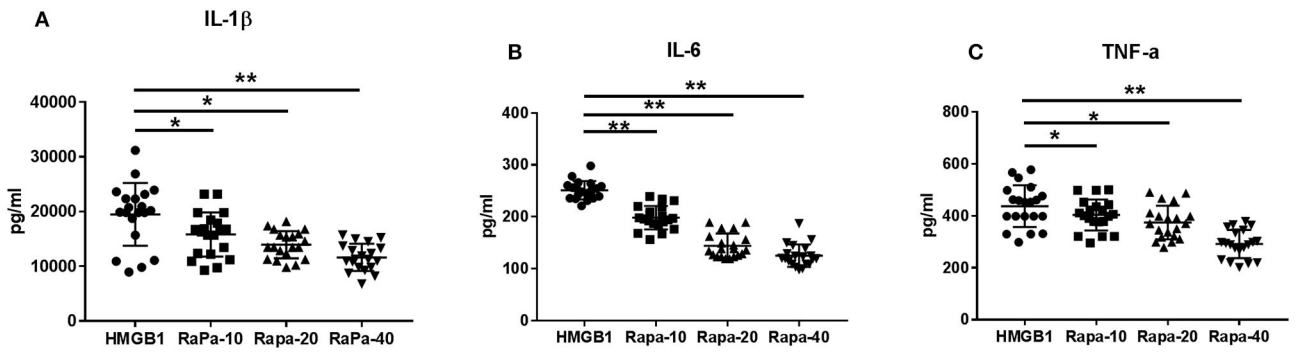

FIGURE 8 | Cytokine production through Rapa intervention. mDCs from systemic lupus erythematosus (SLE) patients were blocked by different concentrations of rapamycin, and production of IL-1β **(A)**, IL-6 **(B)**, and TNF-α **(C)** were measured by ELISA. Different concentrations of rapamycin (Rapa-10: 10 ng/ml; Rapa-20: 20 ng/ml; Rapa-40: 40 ng/ml) were added into cultures of primary mDCs. Data are presented as the median and interrogative range. $^{*}p < 0.05$, $^{**}p < 0.01$. IL, interleukin; TNF, tumor necrosis factor.

There are some limitations to our research. First, all the patients in the study had moderate severity of SLE. Thus, the findings may not be generalizable to patients with mild SLE. Second, we used HMGB1 to stimulate myeloid-derived dendritic cells (mDCs). However, mature mDCs cannot be separated directly from peripheral blood. Instead, PBMCs were isolated and induced to mature mDCs with the cytokine. The mDCs in this study shared some similar characteristics with those in peripheral blood. The degree of similarity between these mDCs cultured *in vitro* and those *in vivo* warrants further investigation. Lastly, the effect of HMGB1- activated dendritic cells on T cells was not evaluated, which is needed further investigation. However, HLA-DR and costimulatory molecules of dendritic cells and cytokine synthesis were measured to estimate the activation of dendritic cells.

In summary, our study found that the mDCs were activated in the peripheral blood of SLE. HMGB1 induces dendritic cell activation and up-regulate the mTOR pathway in SLE. Rapamycin can inhibit HMGB1-induced activation of mDCs and secretion of pro-inflammatory cytokines. These findings indicate that targeting mTOR pathway could be a novel therapeutic approach to SLE.

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 Medical Ethical Committee of the First Affiliated Hospital of Guangxi Medical University. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

XS, HZ, YZ, YL, and QT conducted the study and collected the data. XS, XiaZ, and HZ were involved in study design, data analyses, and writing and editing of 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/fmed.2021.636188/full#supplementary-material

Supplementary Figure 1 | CD11c + DC purity tests before and after cell culture. (**A**: CD11c + DC purity tests before cell culture; **B**: CD11c + DC purity tests before cell culture).

Supplementary Table 1 | Characteristics of systemic lupus erythematosus (SLE) patients and healthy control subjects.

Supplementary Table 2 | mTOR and its substrate P70S6K and 4 ebp1 mRNA expression on dendritic cells through Rapa intervention.

Supplementary Table 3 | HLA-DR, CD40, and CD86 in dendritic cells supernatant through Rapa intervention (%).

Supplementary Table 4 | Cytokines in dendritic cells supernatant through Rapa intervention (pg/ml).

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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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STAT3 but Not STAT5 Contributes to the Protective Effect of Electroacupuncture Against Myocardial Ischemia/Reperfusion Injury in Mice

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Electroacupuncture (EA) can help reduce infarct size and injury resulting from myocardial ischemia/reperfusion (I/R); however, the underlying molecular mechanism remains unknown. We previously reported that STAT5 plays a critical role in the cardioprotective effect of remote ischemic preconditioning (RIPC). Here, we assessed the effects of electroacupuncture pretreatment (EAP) on myocardial I/R injury in the presence and/or absence of *Stat5* in mice and investigated whether EAP exerts its cardioprotective effects in a STAT5-dependent manner. Adult *Stat5*$^{fl/fl}$ and *Stat5*-cKO mice were exposed to EAP at Neiguan (PC6) for 7 days before the induction of I/R injury by left anterior descending (LAD) coronary artery ligation. The myocardial infarct size (IS), area at risk, and apoptotic rate of cardiomyocytes were detected. RT-qPCR and western blotting were used to measure gene and protein expression, respectively, in homogenized heart tissues. RNA-seq was used to identify candidate genes and pathways. Our results showed that EAP decreased IS and the rate of cardiomyocyte apoptosis. We further found that STAT5 was activated by EAP in *Stat5*$^{fl/fl}$ mice but not in *Stat5*-cKO mice, whereas the opposite was observed for STAT3. Following EAP, the levels of the antiapoptotic proteins Bcl-xL, Bcl-2, and p-AKT were increased in the presence of *Stat5*, while that of interleukin 10 (IL-10) was increased in both *Stat5*$^{fl/fl}$ and *Stat5*-cKO. The gene expression profile in heart tissues was different between *Stat5*$^{fl/fl}$ and the *Stat5*-cKO mice with EAP. Importantly, the top 30 DEGs under EAP in the *Stat5*-cKO mice were enriched in the IL-6/STAT3 signaling pathway. Our results revealed for the first time that the protective effect of EAP following myocardial I/R injury was attributable to, but not dependent on, STAT5. Additionally, we found that EAP could activate STAT3 signaling in the absence of the *Stat5* gene, and could also activate antiapoptotic, survival, and anti-inflammatory signaling pathways.

Keywords: myocardial ischemia reperfusion, STAT5, STAT3, electro-acupuncture, cardioprotection

INTRODUCTION

Electroacupuncture (EA) is based on acupuncture, a key component of traditional Chinese medicine. Numerous studies have demonstrated that EA is effective as an alternative protective treatment against myocardial ischemia/reperfusion (I/R) injury *via* electrical stimulation at specific acupoints (1–7). Recently, a clinical trial was undertaken to assess the effect of acupuncture treatment on a total of 1,651 patients with chronic stable angina. The results indicated that acupuncture, used as adjunctive therapy, could alleviate pain, reduce anxiety and depression, and improve the quality of life of the patients (5). Additionally, several studies have demonstrated the effectiveness of EA in treating cardiovascular diseases, and revealed some of the mechanisms underlying its effects. These include improving neurological function, modulating humor states (3, 8–11), regulating apoptosis (12–15), reducing calcium overload and antioxidative stress (16–19), activating anti-inflammatory pathways (12, 20), promoting angiogenesis (21), and regulating energy metabolism (22). However, the fundamental molecular mechanisms involved in the cardioprotective effect of EA have yet to be identified.

There is evidence that EAP can protect the ischemic myocardium against I/R injury (7, 14, 22, 23). While RIPC has been applied as one of common cardioprotective strategies against I/R injury (4, 24, 25), EAP is considered functionally similar to transcutaneous electrical nerve stimulation (TENS) and RIPC (4, 26). Studies on ischemic conditioning have been undertaken using different species, such as mice, rats, pigs, and humans (27–34). The most practical model of myocardial ischemia involves coronary occlusion, leading to the partial or complete obstruction of blood flow in a coronary artery, which mimics the clinical signs of coronary heart disease. Ideally, RIPC or EA pretreatment experiments on treating heart disease should be carried out using big animals or human patients as models (35, 36), whereas mechanistic studies are better performed on small animals, especially when a knockout model is needed. Additionally, evidence from both human patients and mice has indicated that STAT5 plays an important role in RIPC (27, 33, 37). Given that there are many similarities between EAP and RIPC, we therefore use the Stat5-knockout mice model to study the protection of EAP from myocardial I/R injury and its underlying mechanism.

We established a myocardial I/R mouse model using cardiomyocyte-specific *Stat5*-knockout (*Stat5*-cKO) mice. EA was applied to the mice 7 days before surgery to induce I/R injury. We also undertook genome-wide gene profiling to identify candidate genes involved in the cardioprotective role of EAP, and detected some functional pathways.

MATERIALS AND METHODS

Mice

Stat5-floxed mice ($Stat5^{fl/fl}$), generated as previously described (37), were a kind gift from Dr. Hennighausen (NIDDK, NIH). *Tnnt2*-Cre male mice (*Tnnt2Cre*) were a gift from Bin Zhou (Shanghai Institutes for Biological Sciences of the Chinese Academy of Sciences). *Stat5*-cKO mice were generated by crossing these two genotypes. Doxycycline hyclate (Sigma-Aldrich) was added to the drinking water of mice at a concentration of 2 mg/mL and administered for 7 days. Genotyping was performed as previously described (37).

Study Groups

The mice were randomly divided into the following four groups: $Stat5^{fl/fl}$+I/R, $Stat5^{fl/fl}$+EA+I/R, *Stat5*-cKO+I/R, and *Stat5*-cKO+EA+I/R. The $Stat5^{fl/fl}$+I/R and *Stat5*-cKO+I/R mice were exposed to LAD coronary artery occlusion for 30 min, and then reperfused for 180 min, while the $Stat5^{fl/fl}$+EA+I/R and *Stat5*-cKO+EA+I/R mice were subjected to EAP treatment 7 days before LAD artery ligation. All animal studies were carried out according to Chinese and international guidelines for the experimental use of animals. All experiments were approved by the Institute for Animal Care and Use Committee at Nanjing University of Chinese Medicine.

In vivo Experiments

EA was performed at bilateral PC6 (also called Neiguan) acupoints in the $Stat5^{fl/fl}$+EA+I/R and *Stat5*-cKO+EA+I/R mice as previously described (12). The PC6 acupoints are located in the anteromedial aspect of the forelimb between the radius and ulna, 3-mm proximal to the wrist joints (12, 38). Anesthesia was induced with 5% isoflurane and maintained with 1–2% isoflurane in pure oxygen. Sterilized disposable stainless steel acupuncture needles (0.18 mm × 13 mm, Beijing Zhongyan Taihe Medical Instruments Factory, Beijing, China) were inserted into the muscle layer ∼1–2 mm below bilateral PC6 simultaneously using Han's EA instrument (Han Acuten, WQ1002F, Beijing, China). The frequency was 2/15 Hz (alternating dense and disperse mode) and the intensity was 0.5–1.0 mA. Stimulation was applied for 20 min once a day for a total of 7 days. The mice in the $Stat5^{fl/fl}$+I/R and the *Stat5*-cKO+I/R groups were restrained for 20 min without EA stimulation. The $Stat5^{fl/fl}$+I/R and *Stat5*-cKO+I/R mice (control groups) were also anesthetized daily for 7 days before I/R surgery.

The I/R operation was performed according to a previously described protocol (12, 37). Briefly, all the mice were anesthetized by 5% isoflurane and anesthesia was then maintained with 2% isoflurane in a mixture of 70% N_2O and 30% O_2. Under anesthesia, the mice were subjected to a left thoracotomy and LAD artery ligation. Ischemia was confirmed by myocardium blanching, as well as ST-segment elevation and widening of the QRS complex in ECG (37). After 30 min, reperfusion was performed by quickly releasing and removing the suture continuously for 3 h. In the sham-operation group, the same procedure was performed except for the LAD artery ligation. Mice were euthanized by cervical dislocation and the heart specimens were harvested.

Determination of Infarct Size

After harvesting, the hearts were injected for 1–2 min with 0.2 mL of 2% Evans blue dye into the ventricle as previously described (39, 40). The excised and frozen hearts were quickly

sliced into five pieces, placed in 2 mL of 1% TTC (Sigma-Aldrich, St. Louis, MO, USA) in phosphate-buffered saline (PBS), and incubated at 37°C for 15 min. After incubating, the sections were placed in 4% (*v*/*v*) paraformaldehyde at 4°C for 12 h. Unaffected myocardial tissue was stained blue, while the area at risk (AAR) and the infarcted area were unstained and showed as red or white. The infarcted area, AAR, and total left ventricular (LV) area were quantified using Image-Pro Plus 6.0 software (NIH, USA). The infarct size (IS) and AAR percentages were calculated using the following formulas: IS (%) = IS/AAR × 100; AAR (%) = AAR/total LV area × 100 (39, 40).

Apoptosis Measurements

TUNEL staining was used to detect cell apoptosis in cardiac tissue in each group. All the protocols were performed as previously described (37). Heart tissues were harvested and embedded in optimal cutting temperature (OCT) compound (Thermo Scientific, USA). Then, 8-μm-thick tissues were subjected to TUNEL staining according to the instructions of the manufacturer (Cat no. 11684817910, Roche Diagnostics, Lewes, UK). Ten sections were randomly selected from at least 3 animals per group and visualized using a fluorescence microscope (Nikon, Japan). DNase-I served as the positive control labeling solution as the negative control.

Western Blotting

Whole-ventricle samples were lysed with RIPA buffer containing protease and phosphatase inhibitors based on the Protease Inhibitor Cocktail (100X) (Thermo Scientific, USA). Homogenates were centrifuged at 14,000 × g for 10 min at 4°C, and the collected supernatants were stored at −80°C until further use. Protein concentrations were determined using a BCA protein assay (Pierce, Thermo Scientific, USA). Protein was mixed with 5× Laemmli loading buffer and heated at 95°C for 10 min. Equal amounts of protein were subjected to SDS–PAGE and transferred to polyvinylidene fluoride membranes. The samples were incubated with primary antibodies against Bcl-xL (1:1,000, Cell Signaling Technology, #2762), Bcl-2 (1:1,000, Cell Signaling Technology, #3498), Cyt c (1:1,000, Cell Signaling Technology, #4280), phospho-STAT5 (1:1,000, Cell Signaling Technology, #4322), STAT5 (1:1,000, Cell Signaling Technology, #94205), phospho-STAT3 (1:1,000, Cell Signaling Technology, #4093), STAT3 (1:1,000, Cell Signaling Technology, #4904), phospho-AKT (1:1,000, Cell Signaling Technology, #4060), AKT (1:1,000, Cell Signaling Technology, #4298), IL-10 (1:1,000, Abcam, #ab192271), VEGFA (1:1,000, Abcam, #ab46154), beta-actin (1:1,000, Abcam, #ab8226), or GAPDH (1:1,000, Cell Signaling Technology, #2118) overnight at 4°C, and then with a secondary antibody for 2 h at room temperature. Immunoreactive bands were revealed using SuperSignal West Pico Chemiluminescent Substrate (Pierce) and quantified using the ChemiDoc Imaging System (Bio-Rad).

Quantitative Reverse Transcription PCR

Total RNA was extracted from heart tissue using TRIzol reagent (Invitrogen, Mannheim, Germany), and reverse-transcribed to cDNA using reverse transcriptase and random primers (11121ES60, Yeasen Biotech Co., Ltd., China). Target genes were amplified on a MX3000P thermocycler (Stratagene, La Jolla, CA, USA) using SYBR Green (Q431-02, Vazyme Co., China). Gene expression was quantified using the $2^{-\Delta\Delta Ct}$ method. The primer sequences were as follows: *Il6*, GACTTCACAGAGGATAC CACCC (forward) and GACTTCACAGAGGATACCACCC (reverse); *gp130*, GAGCTTCGAGCCATCCGGGC (forward) and AAGTTCGAGCCGCGCTGGAC (reverse); beta-actin, GGTGAAGACGCCAGTAGAC (forward) and TGCTGGAAG GTGGACAGTGA (reverse).

RNA Sequencing Analysis

RNA-seq for mouse heart tissues was performed using the Illumina Hiseq 2500 and 2000 platform (Illumina, USA) as described in our previous study (41). Data analysis was performed as previously described (41). The quality of the raw sequencing data was assessed by FastQC. The Cufflinks and Cuffdiff programs were used to assemble individual transcripts and for differential transcript expression analysis, respectively. The pathways were analyzed using DAVID Bioinformatics Resources. Genes with fewer than 1.0 fragments per kilobase of exon per million fragments mapped (FPKM) were filtered out. Log2 fold change (FC) $\geq|\pm 1|$ and $P < 0.05$ were used as thresholds for identifying upregulated and downregulated genes.

Statistical Analysis

Data analyses and treatment conditions were double-blinded. SPSS 18.0 software was used for statistical analysis. All data were expressed as means ± standard error of the mean (SEM). A two-tailed unpaired Student's *t*-test was used for comparisons between two groups. For comparisons between multiple groups, one-way or two-way ANOVA was used followed by the Bonferroni *post-hoc* test when equal variances were assumed. $P < 0.05$ was considered statistically significant.

RESULTS

EAP Reduced Myocardial Infarct Size and Attenuated Cardiomyocytic Apoptosis to the Same Extent in Both *Stat5*$^{fl/fl}$ and *Stat5*-cKO Mice

EAP had no effect on the daily behavior or cardiac performance of mice of either genotype. After myocardial I/R surgery, we harvested the heart tissues and measured myocardial infarct areas and AARs (**Figure 1**). We found that EAP significantly reduced infarct size in both *Stat5*$^{fl/fl}$ mice (55.2 ± 10.8% without EAP vs. 28.6 ± 4.1% with EAP, $P < 0.01$) and *Stat5*-cKO mice (65.5 ± 5.3% without EAP vs. 29.6 ± 9.6% with EAP, $P < 0.01$). No significant difference in AAR was seen between the *Stat5*$^{fl/fl}$+EA+I/R and the *Stat5*-cKO+EA+I/R mice.

TUNEL staining was performed to detect apoptosis in myocardial cells. As shown in **Figure 2**, mice in the

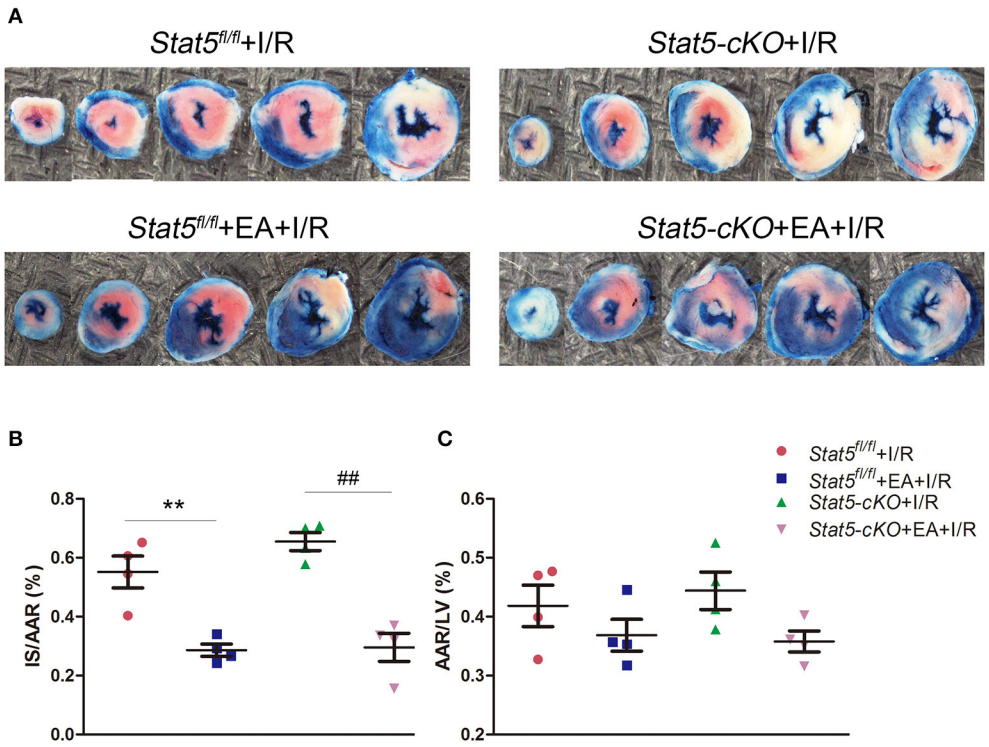

FIGURE 1 | Acupuncture reduced myocardial infarct size. **(A)** Evans blue/TTC double staining was used to measure the ischemic infarct area and area at risk (AAR). **(B)** The infarct size (IS)/AAR ratio was calculated and presented as a percentage. Data are presented as means ± SEM. Normal tissues are stained blue, ischemic infarct areas and AARs are pale white or red. $^{**}P < 0.01$ compared with *Stat5*$^{fl/fl}$+I/R group; $^{\#\#}P < 0.01$ compared with *Stat5*-cKO+I/R group. **(C)** The ratio of AAR/total left ventricular (LV) area was calculated and presented as a percentage. There was no difference between the groups. Data were analyzed by one-way ANOVA with Tukey's *post-hoc* correction, $n = 4$.

Stat5$^{fl/fl}$+EA+I/R group had fewer TUNEL-positive cells compared with those in the *Stat5*$^{fl/fl}$+I/R group (1.85 ± 0.26% vs. 5.62 ± 0.56%, $P < 0.01$). Similarly, the number of apoptotic myocardial cells was significantly lower in mice of the *Stat5*-cKO+EA+I/R group than in those of the *Stat5*-cKO+I/R group (1.85 ± 0.32 vs. 5.83 ± 0.35%, $P < 0.01$).

EAP Activated STAT5 in *Stat5*$^{fl/fl}$ Mice, but Not in *Stat5*-cKO Mice, Following Myocardial I/R Surgery

To further explore whether the myocardial protective effect of EAP against I/R injury is STAT5-dependent, we examined the expression of p-STAT5 in heart tissues by western blotting. EAP markedly increased the protein levels of p-STAT5/GAPDH in *Stat5*$^{fl/fl}$ mice compared with those in *Stat5*$^{fl/fl}$ mice subjected to I/R; however, EAP did not affect STAT5 activation in the hearts of *Stat5*-cKO mice (**Figures 3A,B**). This suggested that STAT5 may be involved in the EAP-mediated protective effects against myocardial I/R injury.

EAP Activated IL-6/gp130/STAT3 Signaling in the Absence of *Stat5*

Given that STAT3 might compensate for the loss of STAT5, we then evaluated the STAT3 and p-STAT3 protein expression levels in the heart tissues of both *Stat5*$^{fl/fl}$ and *Stat5*-cKO mice. The results showed that the expression of p-STAT3 was increased in *Stat5*-cKO+EA+I/R mice compared with that in mice of the *Stat5*-cKO+I/R group; however, this was not observed in *Stat5*$^{fl/fl}$ mice (**Figure 4A**). To understand the mechanism by which STAT3 was activated in this process, we further assessed the expression levels of genes acting upstream of STAT3. We found that the mRNA expression of *Il6* and *gp130* was greatly increased in *Stat5*-cKO+EA+I/R mice compared with that in *Stat5*-cKO+I/R mice; however, these effects were not observed in the presence of *Stat5*. This suggested that, in the absence of the *Stat5* gene, EAP may activate the IL-6/gp130/STAT3 pathway at the mRNA level when the heart is exposed to myocardial I/R injury (**Figure 4B**).

Genome-Wide Analysis Revealed the Gene Expression Profiles in Both *Stat5*$^{fl/fl}$ and *Stat5*-cKO Mice With or Without EAP Followed by Myocardial I/R Injury

To identify genes that may have a role in EAP-mediated protection against myocardial I/R injury, RNA was extracted from the heart tissues for RNA-seq analysis. The Cufflinks package was used to filter out the top 30 differentially expressed genes (DEGs) between the *Stat5*$^{fl/fl}$+I/R and *Stat5*$^{fl/fl}$+EA+I/R

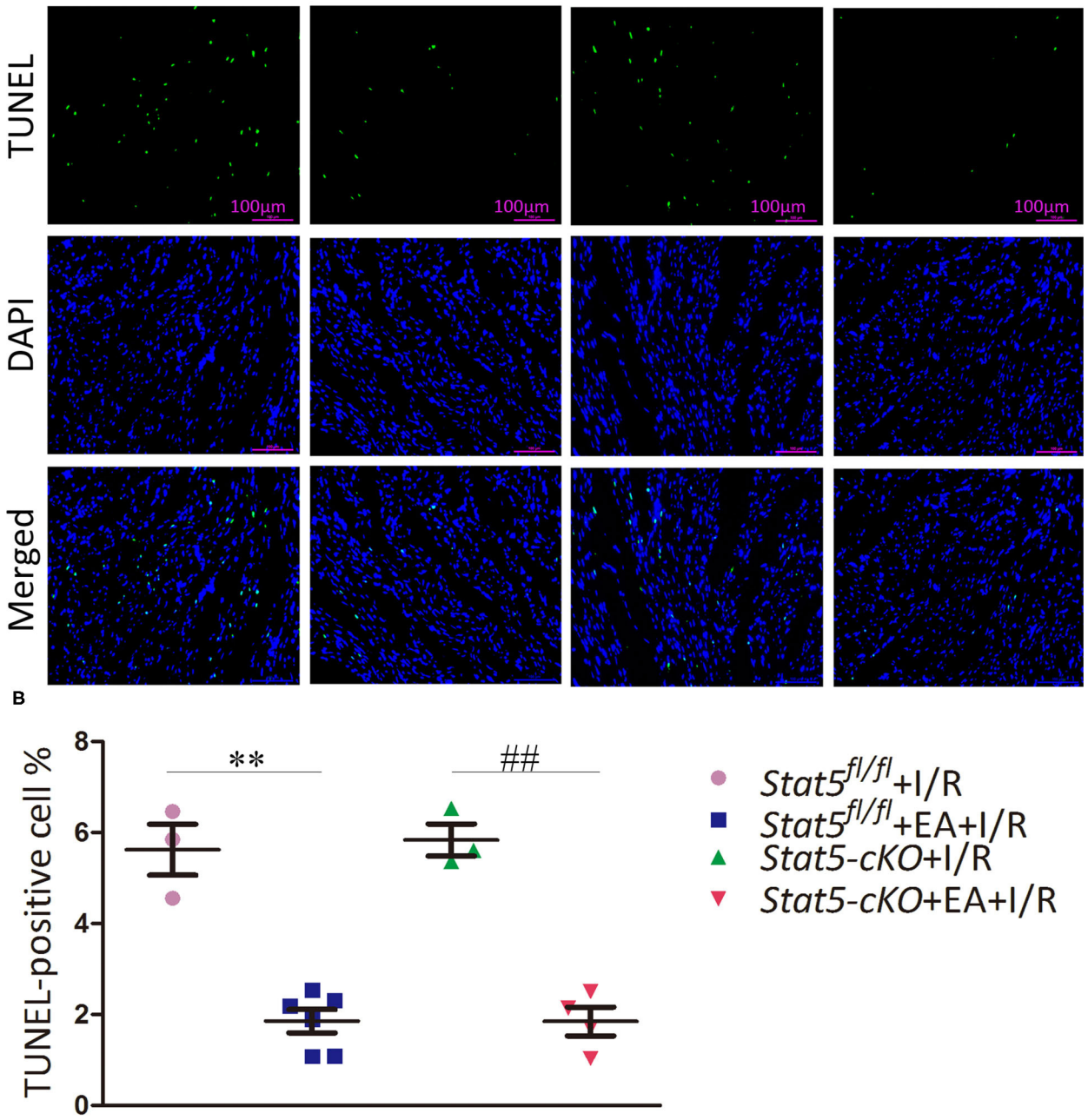

FIGURE 2 | The effects of electroacupuncture pretreatment (EAP) on apoptosis in myocardial tissues in the *Stat5*$^{fl/fl}$ and the *Stat5*-cKO mice. **(A,B)** The rate of apoptosis was measured by TUNEL staining. Values are means ± SEM. $^{**}P < 0.01$ compared with the *Stat5*$^{fl/fl}$+I/R group; $^{\#\#}P < 0.01$ compared with the *Stat5*-cKO+I/R group. Data were analyzed by two-way ANOVA with Bonferroni's multiple comparison test, $n = 3$–6.

groups and the *Stat5*-cKO+I/R and *Stat5*-cKO+EA+I/R groups (**Tables 1A,B**). Venn diagrams were drawn based on the list of filtered DEGs among these four groups (**Figure 5**). The results showed that 1,052 genes were differentially expressed between the *Stat5*$^{fl/fl}$+I/R and *Stat5*$^{fl/fl}$+EA+I/R groups, while 1,039 genes were found to be differentially expressed between the *Stat5*-cKO+I/R and *Stat5*-cKO+EA+I/R groups; of these DEGs, 133 overlapped between these two clusters (**Figure 5**). Among the four groups, only two genes, *Hspa1a* and *Pttg1*, were found to be differentially expressed in all the groups (**Table 1**).

To further understand the potential pathways involved in the regulation of STAT5-related DEGs and that of EAP-related DEGs

TABLE 1 | The top 30 differentially expressed genes with a log2 (FC) > |±1|and $q < 0.05$.

Up-regulated in EA against I/R				Down-regulated in EA against I/R			
Gene name	**Value_1**	**Value_2**	**log2 (fold_change)**	**Gene name**	**Value_1**	**Value_2**	**log2 (fold_change)**
A. The top 30 differentially expressed genes obtained from comparing *Stat5*$^{fl/fl}$+EA+I/R vs. *Stat5*$^{fl/fl}$+I/R							
Fosb	0.181422	27.5826	7.24827	Hbb-bt	35.5975	1.35656	−4.71375
Retnlg	1.26857	106.281	6.38853	Tcf15	53.5841	3.7788	−3.82581
Crisp1	1.13838	82.4984	6.17931	Ccn5	6.67781	0.624545	−3.4185
Fos	1.59922	114.642	6.16363	Myl4	60.998	5.99943	−3.34586
Cxcl5	0.375871	21.1825	5.81649	Scand1	105.37	11.7828	−3.16071
Selp	0.314312	16.2764	5.69443	Zhx2	1.93407	0.220615	−3.13204
Cxcl1	1.96399	95.2442	5.59977	Nrtn	43.6184	5.0225	−3.11846
S100a8	5.82634	256.694	5.46131	Tnfrsf25	3.90054	0.503182	−2.95452
Atf3	2.46681	106.299	5.42933	Pttg1	33.8997	4.94227	−2.77803
Ptx3	0.51755	19.8976	5.26476	Zfp771	19.5158	3.02169	−2.69122
Nr4a3	0.472838	17.3526	5.19767	Fzd2	3.1422	0.491571	−2.6763
Sele	0.190666	6.83815	5.16449	Fxyd3	3.78128	0.621019	−2.60617
Socs3	2.33823	83.0272	5.1501	Aplnr	15.2807	2.89142	−2.40186
Egr1	3.52759	122.674	5.12	Cited4	17.5033	3.57884	−2.29006
S100a9	11.0427	381.325	5.10986	Eva1b	18.5641	3.80599	−2.28617
Il18rap	0.0722091	2.21294	4.93764	Msx1	3.34857	0.6924	−2.27387
Thbs1	1.59068	41.5123	4.70582	Dkk3	5.70321	1.21389	−2.23213
Rdh12	0.122831	3.01114	4.61557	Rnaset2a	22.5668	4.88218	−2.2086
Hspa1b	2.40171	58.418	4.60428	Ifi27l2a	180.402	39.5256	−2.19036
Hspa1a	1.99294	44.6613	4.48606	Nrarp	11.2641	2.52363	−2.15815
Adam8	0.400678	8.43168	4.39531	Kctd15	2.65098	0.611701	−2.11563
Ch25h	0.769351	15.8707	4.36658	Hic1	7.47804	1.74196	−2.10195
Nts	0.799365	15.3258	4.26096	Gas1	16.0842	3.82293	−2.07289
Ifitm6	1.20354	22.6423	4.23367	Oas1a	3.5286	0.843947	−2.06387
Egr2	0.16011	3.01005	4.23265	Dynll1	78.6361	18.9174	−2.05548
Arc	1.14086	20.7638	4.18587	Trim47	26.4865	6.40037	−2.04903
Agt	1.55521	27.442	4.1412	Tmsb10	110.513	26.7491	−2.04665
Rnd1	0.793702	12.8902	4.02154	B3gnt3	2.39685	0.580215	−2.04648
Pdk4	35.6426	578.857	4.02153	Myo7a	2.29499	0.574977	−1.99691
Plaur	1.59226	25.5567	4.00455	Fam181b	3.87361	0.976908	−1.98738
B. The top 30 differentially expressed genes obtained from comparing *Stat5-cKO*+EA+I/R vs. *Stat5-cKO*+I/R.							
Eno1b	0.503646	6.53728	3.69821	Olfr1033	855.24	3.24857	−8.04038
Dynlt1b	1.11668	14.442	3.69299	Gm45551	221.558	1.31604	−7.39534
H2-Q1	0.264109	3.03007	3.52015	Gm38271	30.2569	0.353232	−6.4205
Tmem181c-ps	0.99792	11.1342	3.47994	Psg16	2.41103	0.0719611	−5.06629
Gm4737	0.445121	4.58914	3.36595	Gm3365	7.26917	0.262255	−4.79275
2610005L07Rik	1.25086	12.1968	3.28551	Sugct	11.7515	0.52977	−4.47133
Gm14421	0.377049	3.17075	3.072	Gm43197	55.389	3.11501	−4.15229
Mmp3	0.379936	3.17412	3.06253	Gm15280	32.1097	2.05499	−3.96581
Gm42887	0.544175	4.41682	3.02087	CAAA01147332.1	97.5278	7.86365	−3.63254
Ubb	35.8823	290.433	3.01686	Zfp729a	6.97773	0.621896	−3.48801
Hba-a2	44.9947	363.254	3.01315	Adgra3	25.8651	3.26536	−2.98569
Tmem191c	0.28056	2.17679	2.95582	Fmod	3.18759	0.452927	−2.81512
Gdnf	0.160275	1.21115	2.91776	Dpy19l3	2.2277	0.347531	−2.68034
Rpl3-ps1	3.02696	22.6124	2.90117	Gm37324	1.86878	0.299513	−2.64141
Stbd1	0.465139	3.32577	2.83795	Gm48274	72.516	11.6454	−2.63854
Adh6b	0.422625	3.00224	2.82859	Pilra	3.10358	0.507	−2.61387
Hba-a1	78.1547	542.904	2.79629	Prc1	1.51292	0.247267	−2.6132
Polr2l	6.80429	43.8946	2.68952	Clec4e	6.2329	1.08171	−2.52658
Myh7	174.331	1091.89	2.64692	Spp1	3.08682	0.543414	−2.506
Gapdh	603.792	3577.93	2.567	Rac2	14.0493	2.49669	−2.49241
Pttg1	5.6626	32.9153	2.53922	Oxnad1	117.772	21.1786	−2.47532
Zc3h3	0.178519	1.03346	2.53333	Fggy	4.18537	0.772849	−2.4371
Gm6472	5.6958	31.1981	2.45349	Gm44215	2.03074	0.376049	−2.43301
Cys1	0.799506	4.37525	2.45218	Lars2	129.273	25.3961	−2.34774
Tgtp2	0.951374	5.16733	2.44134	Zfp975	3.23415	0.636673	−2.34476
Rps6	94.127	497.199	2.40114	Bace2	8.13204	1.6647	−2.28836
Hspa1a	20.8766	109.614	2.39248	Suds3	101.31	22.7191	−2.1568
Eif3j2	0.89728	4.63881	2.37012	Tesk1	11.7699	2.74535	−2.10004
Gm8116	1.05436	5.39728	2.35587	Insig2	86.0597	20.4798	−2.07113
Gm15459	34.4931	176.148	2.35241	Pnkp	30.3154	7.36189	−2.0419

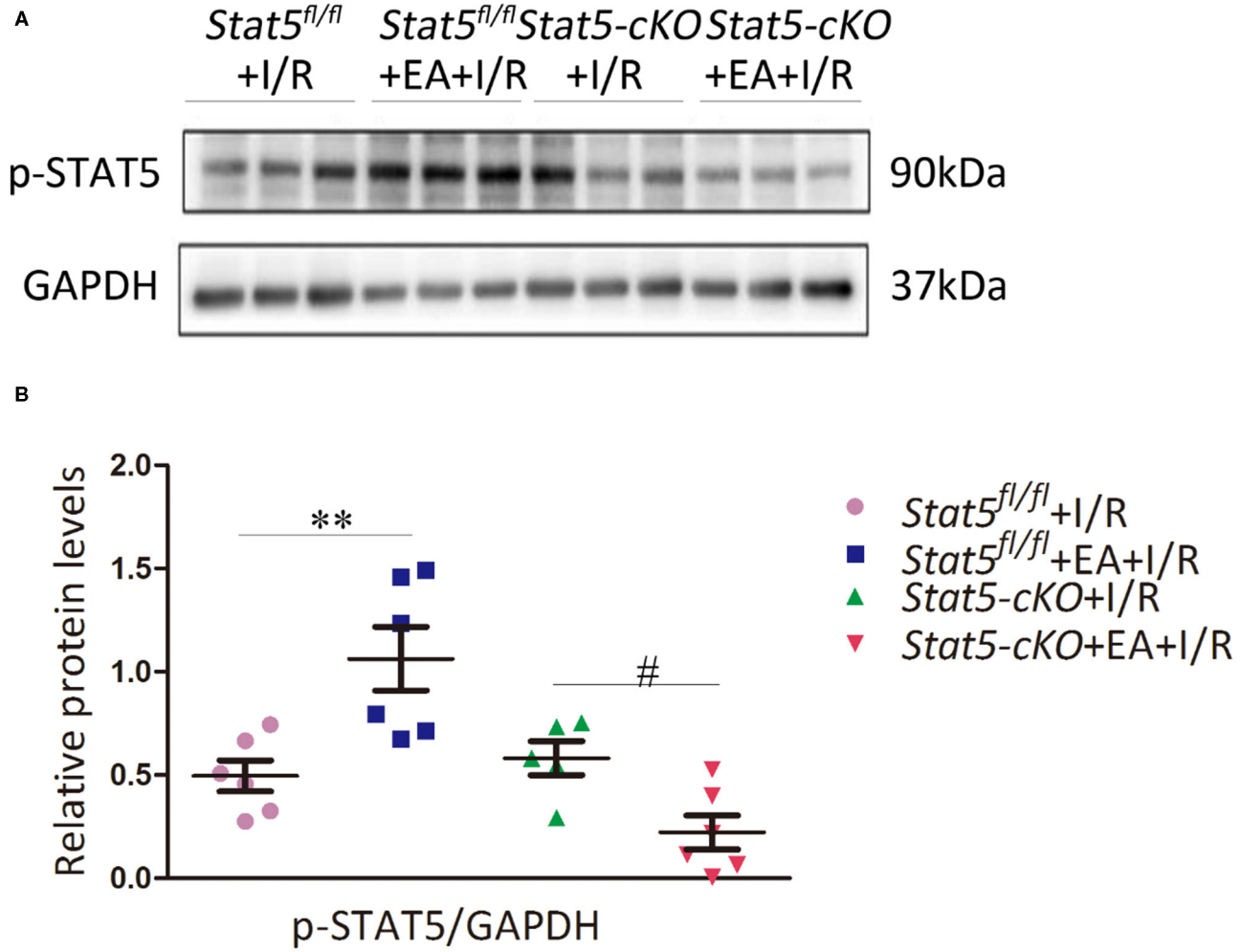

FIGURE 3 | Electroacupuncture pretreatment (EAP) activated the STAT5 protein in the heart tissues of *Stat5*$^{fl/fl}$ mice, but not in those of *Stat5*-cKO mice. **(A)** Representative western blotting images. **(B)** Quantitative analysis of p-STAT5 protein levels in each group. Data are presented as means ± SEM of at least three independent experiments. $^{**}P < 0.01$ compared with the *Stat5*$^{fl/fl}$+I/R group; $^{\#}P < 0.05$ compared with the *Stat5*-cKO+I/R group. Data were analyzed by two-way ANOVA with Bonferroni's multiple comparison test, $n = 6$.

under conditions of I/R injury, we then carried out a pathway analysis for these DEGs using DAVID Bioinformatics Resources. The top 20 pathways are outlined in **Figure 6**.

KEGG pathway analysis suggested that, in the presence of *Stat5*, EAP-activated genes were mainly enriched in the JAK/STAT, TNF, IL-17, NF-κB, and MAPK signaling pathways, as well as in cytokine–cytokine receptor interaction (**Figure 6A**). In contrast, in the *Stat5*-cKO mice, the DEGs associated with EAP-mediated myocardial protection were mainly concentrated in ribosome pathways, thermogenesis, and the oxidative phosphorylation pathway (**Figure 6B**). We also analyzed the top 20 KEGG pathways associated with the 133 overlapping genes (**Figure 6C**) and found that some of the EAP-regulated, STAT5-independent DEGs were mainly linked with inflammation-related pathways such as the IL-7 signaling pathway, human T-cell leukemia virus 1 infection, antigen processing and presentation, and the TNF signaling pathway.

EAP Influenced Apoptotic and Survival Signaling Only in the Presence of STAT5

The genome-wide profiling data indicated that EAP can activate antiapoptotic and survival signaling in mice with I/R injury. To further validate these findings, we investigated the expression of apoptosis- and survival-related proteins in the myocardial tissue of *Stat5*$^{fl/fl}$ and *Stat5*-cKO mice following EAP. The results showed that the expression levels of Bcl-2 and Bcl-xL were significantly increased in the *Stat5*$^{fl/fl}$+EA+I/R group compared with those in the *Stat5*$^{fl/fl}$+I/R group ($P < 0.05$), whereas the expression of Cyt c did not differ between these groups (**Figures 7A,B**). In contrast, no marked changes were observed in the expression levels of these proteins in the hearts of *Stat5*-cKO mice either with or without EAP, suggesting that STAT5 is essential for the EAP-mediated activation of antiapoptotic signaling in the I/R injury condition. We then measured the level of IL-10, an important cytokine in cardioprotection,

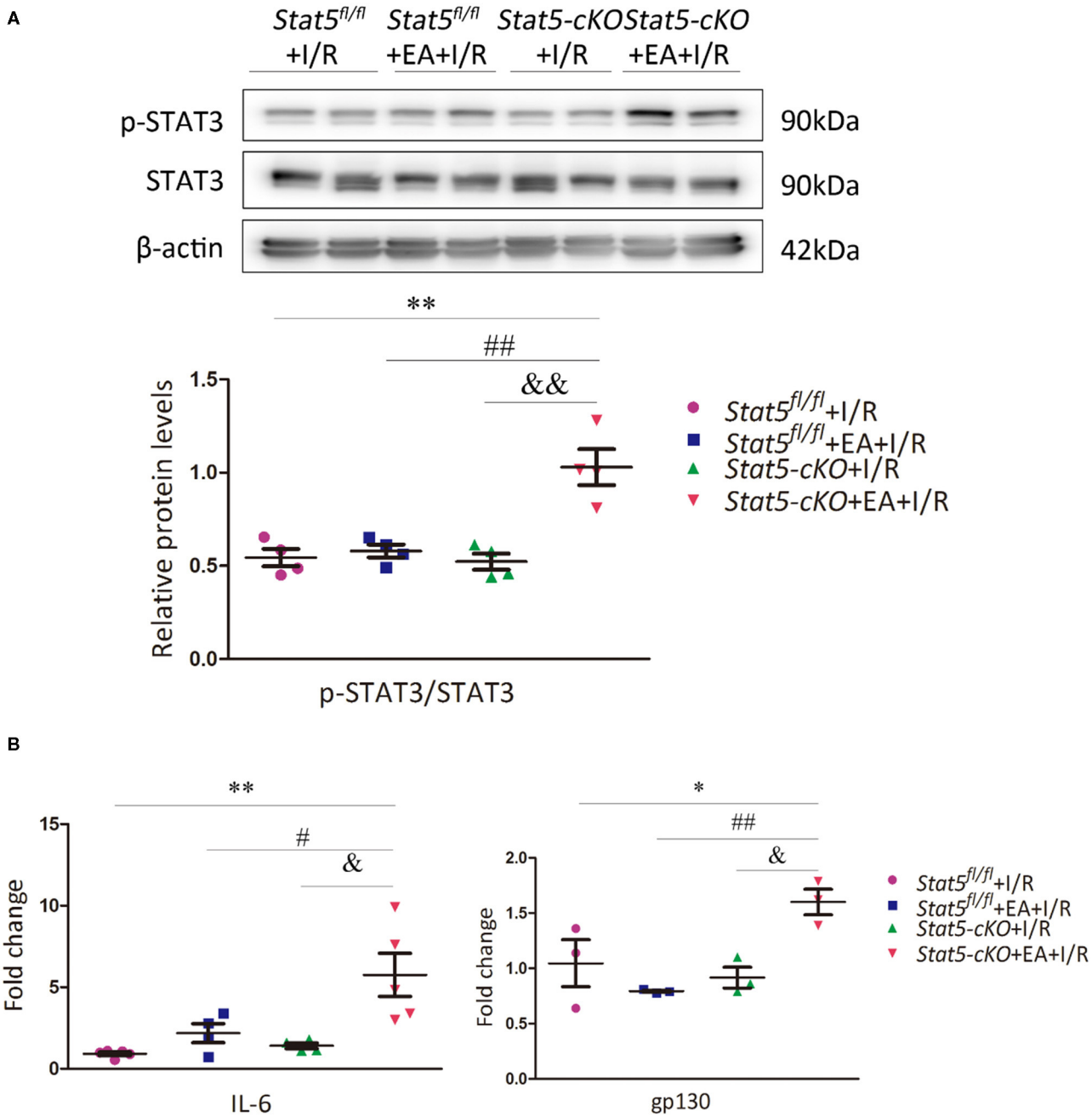

FIGURE 4 | The expression of IL-6/gp130/STAT3 axis-related molecules. **(A)** The protein expression of STAT3 and p-STAT3 was assessed by western blotting. $^{**}P <$ 0.01 compared with the *Stat5*$^{fl/fl}$+I/R group; $^{\#\#}P < 0.01$ compared with the *Stat5*$^{fl/fl}$+EA+I/R group; $^{\&\&}P < 0.01$ compared with the *Stat5*-cKO+I/R, $n = 4$. **(B)** The expression of *Il6* and *gp130* mRNA was measured by RT-qPCR. Data are presented as means ± SEM of at least three independent experiments. $^{*}P < 0.05$, $^{**}P$ < 0.01 compared with the *Stat5*$^{fl/fl}$+I/R group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared with the *Stat5*$^{fl/fl}$+EA+I/R group; $^{\&}P < 0.05$ compared with the *Stat5*-cKO+I/R group. Data were analyzed by two-way ANOVA with Bonferroni's multiple comparison test, $n = 3$–5.

and that of its related proteins PI3K, AKT, and p-AKT (**Figures 8A,B**). The results showed that EAP increased the levels of p-AKT in the presence, but not absence, of STAT5; however, under the same condition, IL-10 was upregulated in the hearts of both *Stat5*$^{fl/fl}$ and *Stat5*-cKO mice. These findings suggested that the EAP-induced activation of survival signaling to protect against myocardial I/R injury was partially STAT5-dependent.

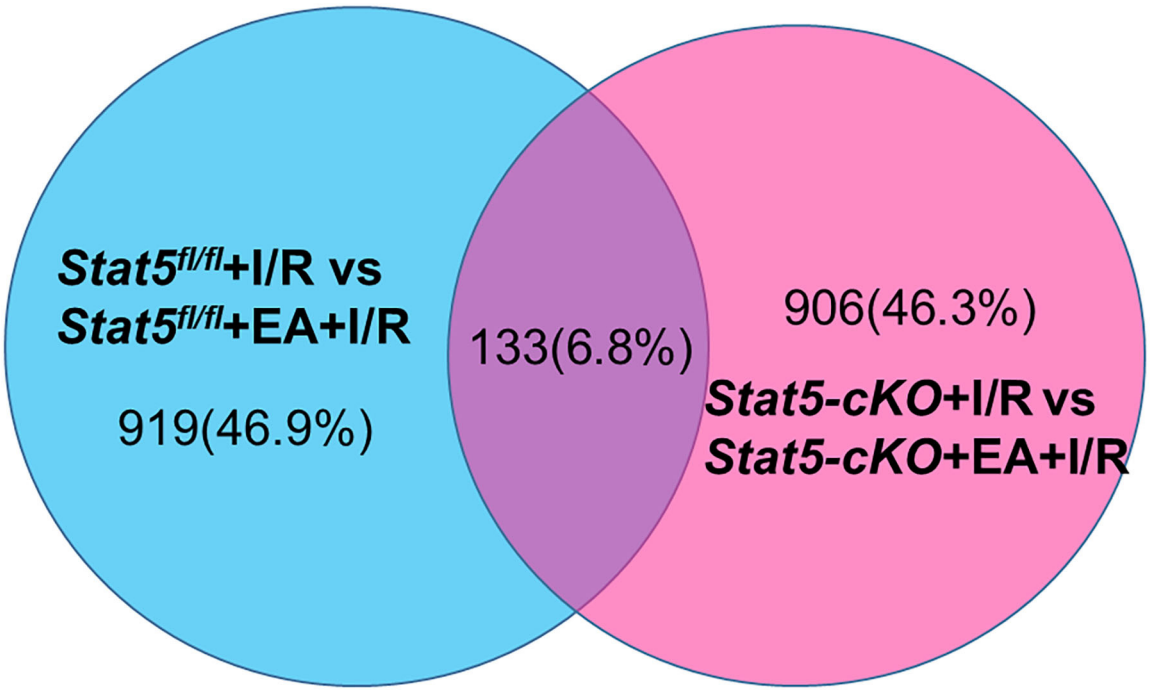

FIGURE 5 | Venn diagrams and clustering analysis of RNA-seq results. Venn diagrams were drawn based on the RNA-seq datasets. The blue circle indicates the numbers of up- and downregulated genes in the *Stat5*$^{fl/fl}$+EA+I/R group *vs*. the *Stat5*$^{fl/fl}$+I/R group the pink circle represents the numbers of up- or downregulated genes in the *Stat5*-cKO+EA+I/R group *vs*. the *Stat5*-cKO+I/R group. A total of 133 genes overlapped between these two clusters.

DISCUSSION

Ischemic heart disease remains the leading cause of premature mortality and disability worldwide (34, 42). Although early coronary reperfusion, a clinically effective method against myocardial I/R injury, can reduce infarct size, reperfusion by revascularization initiates a chain reaction that can promote and amplify post-ischemic injury (43, 44). Pretreatment with EA or RIPC represents a valid method of reducing the risk of myocardial injury (3, 6, 45, 46). In our previous study, we found that STAT5 has a significant impact on RIPC-mediated late cardioprotection through regulating antiapoptotic signaling and the PI3K/AKT survival pathway (37). Similar to RIPC, EAP at acupoint PC6 can also help protect the myocardium under certain disease conditions by stimulating multiple functional pathways.

In the present study, we explored the role of STAT5 in EAP-mediated myocardial protection against I/R by employing cardiomyocyte-specific *Stat5*-cKO mice. Surprisingly, we observed that EAP could reduce the infarct size and the levels of myocardial cell apoptosis in both *Stat5*$^{fl/fl}$ and *Stat5*-cKO mice (**Figures 1**, **2**), suggesting that STAT5 is not indispensable for the cardioprotective effect of EAP against myocardial I/R injury. However, EAP activated STAT5 to promote antiapoptotic and AKT-dependent survival signaling in the presence, but not absence, of *Stat5* (**Figures 7A**, **8A**). This was confirmed by the RNA-seq results for the I/R-injured heart tissues, which showed that STAT5-dependent genes and EAP-regulated genes belonged to different categories (**Table 1**, **Figure 6**). Many of the genes regulated by EAP in the presence of *Stat5* (*Stat5*$^{fl/fl}$+I/R group vs. the *Stat5*$^{fl/fl}$+EA+I/R group), such as *Fosb*, *Fos*, *cxcl1*, *Cxcl5*, *Egr1*, *Egr2*, *Nr4a3*, *Socs3*, *Ccn5*, *Myl4*, *Zhx2*, *Dkk3*, and *Dynll1*, have been reported to play a protective role against myocardial I/R injury, cardiac hypertrophy, or hypoxic insult (47–64). Moreover, in the presence of functional STAT5, many of these genes are known to play antiapoptotic, anti-inflammatory, and antioxidative roles, while some are also involved in STAT3/5 signaling (**Table 1A**). These DEGs act in many functional pathways, such as the JAK/STAT, TNF, apoptotic, or NF-κB signaling pathways (**Figure 6A**). Additionally, we found that among the top 30 genes identified as being differentially expressed between the *Stat5*-cKO+EA+I/R and the *Stat5*-cKO+I/R groups when the *Stat5* gene was absent, *Rps6*, *Mmp3*, *Pttg1*, and *Rac2* were closely associated with the IL-6/STAT3 signaling pathway, as previously reported (65–74) (**Table 1B**). Matrix metallopeptidase 3 (*Mmp3*) encodes an extracellular matrix-degrading enzyme (MMP-3) that is closely linked with tissue remodeling, wound repair, and the progression of atherosclerosis (65). Recent findings have indicated that STAT3 binds to the *Mmp3* promoter and promotes its transcription following IL-6 stimulation (75). Pituitary tumor transforming 1 (*Pttg1*) was originally cloned from rat pituitary tumor cells

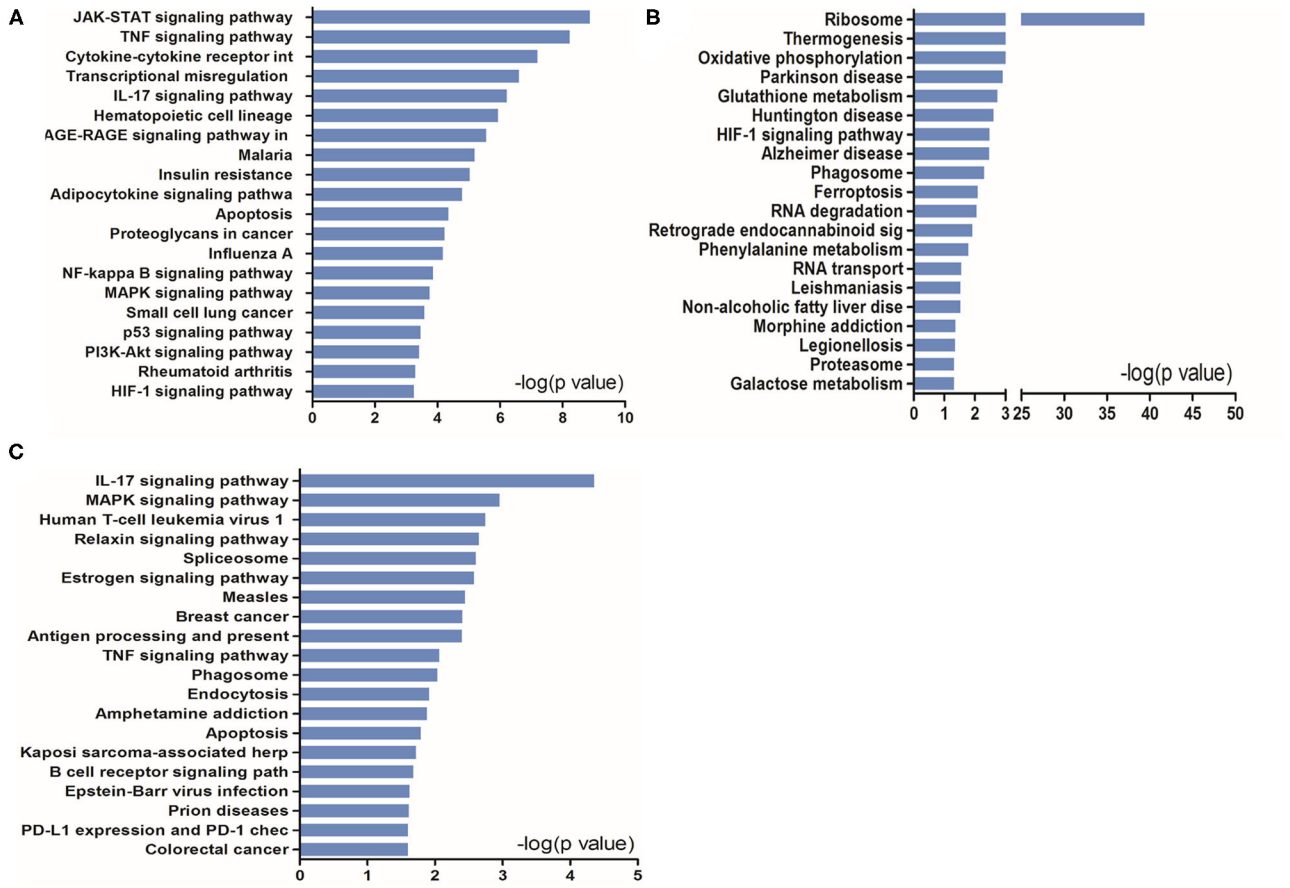

FIGURE 6 | KEGG pathway analysis of up- and downregulated genes in the heart tissues in the presence or absence of *Stat5*. **(A)** The top 20 KEGG pathways were drawn from the 919 differentially expressed genes (DEGs) between the *Stat5*$^{fl/fl}$+I/R group and the *Stat5*$^{fl/fl}$+EA+I/R group shown in **Figure 5**. **(B)** The top 20 KEGG pathways were drawn from the 906 DEGs between the *Stat5*-cKO+I/R group and the *Stat5*-cKO+EA+I/R group shown in **Figure 5**. **(C)** The top 20 KEGG pathways were drawn from the 133 co-regulated genes shown in **Figure 5**.

and was reported to function as an oncogene (76). Huang et al. (70) demonstrated that *Pttg1* expression is regulated by IL-6 *via* the binding of activated STAT3 to the *PTTG1* promoter in LNCa P cells. Rac2, a Rac family member, is mainly expressed in hematopoietic cells. Lai et al. detected that Rac can enhance STAT3 activation and regulate the expression of HIF-2α and VEGF, thereby promoting angiogenesis. The same authors also found that the activation of STAT3, but not STAT5, was reduced in Rac-depleted glioblastoma cells. High levels of intracellular galectin-3 expression are essential for the transcriptional activation of osteopontin [OPN; also known as secreted phosphoprotein 1 (Spp1)] in STAT3-mediated macrophage M2 polarization after myocardial infarction (67, 71). The phosphorylation sites on ribosomal protein S6 (Rps6) have been mapped to five clustered residues, which play an important role in protein synthesis in cardiac myocytes, as well as in cardiac function (66, 72–74). Our KEGG pathway analysis indicated that the DEGs activated by EAP in *Stat5*-cKO mice act mainly in ribosome-related, thermogenesis-related, and oxidative phosphorylation-related pathways (**Figure 6**). Genes involved in the ribosome-related pathway, such as *Rps6* and *Rpl3-ps1*, were markedly upregulated by EAP in mice lacking *Stat5*. Rps6 was reported to be closely related to the IL-6/STAT3 signaling pathway (77, 78). Notably, this pathway has also been linked with mitochondrial function, which is important in cardioprotection (79–81). RNA-seq profiling indicated that the mechanisms underlying the protective effect of EAP against myocardial I/R injury differed between *Stat5*$^{fl/fl}$ and *Stat5*-cKO mice. Combined with our molecular biological data, these results supported that EAP can activate STAT3 in the absence of *Stat5* and help protect against I/R injury.

Multiple studies have demonstrated that in the absence of a given STAT member, receptors will recruit other STAT members instead (82–87). STAT3 and STAT5 show high homology in their functional domains, and have different effects and underlying mechanisms through binding to distinct loci and regulating specific target genes (88). STAT3 and STAT5 proteins can also bind to the same regulatory oncogenic loci, resulting in compensatory or antagonistic signaling (89, 90). Despite the large number of STAT3/STAT5-related studies, the roles of these two

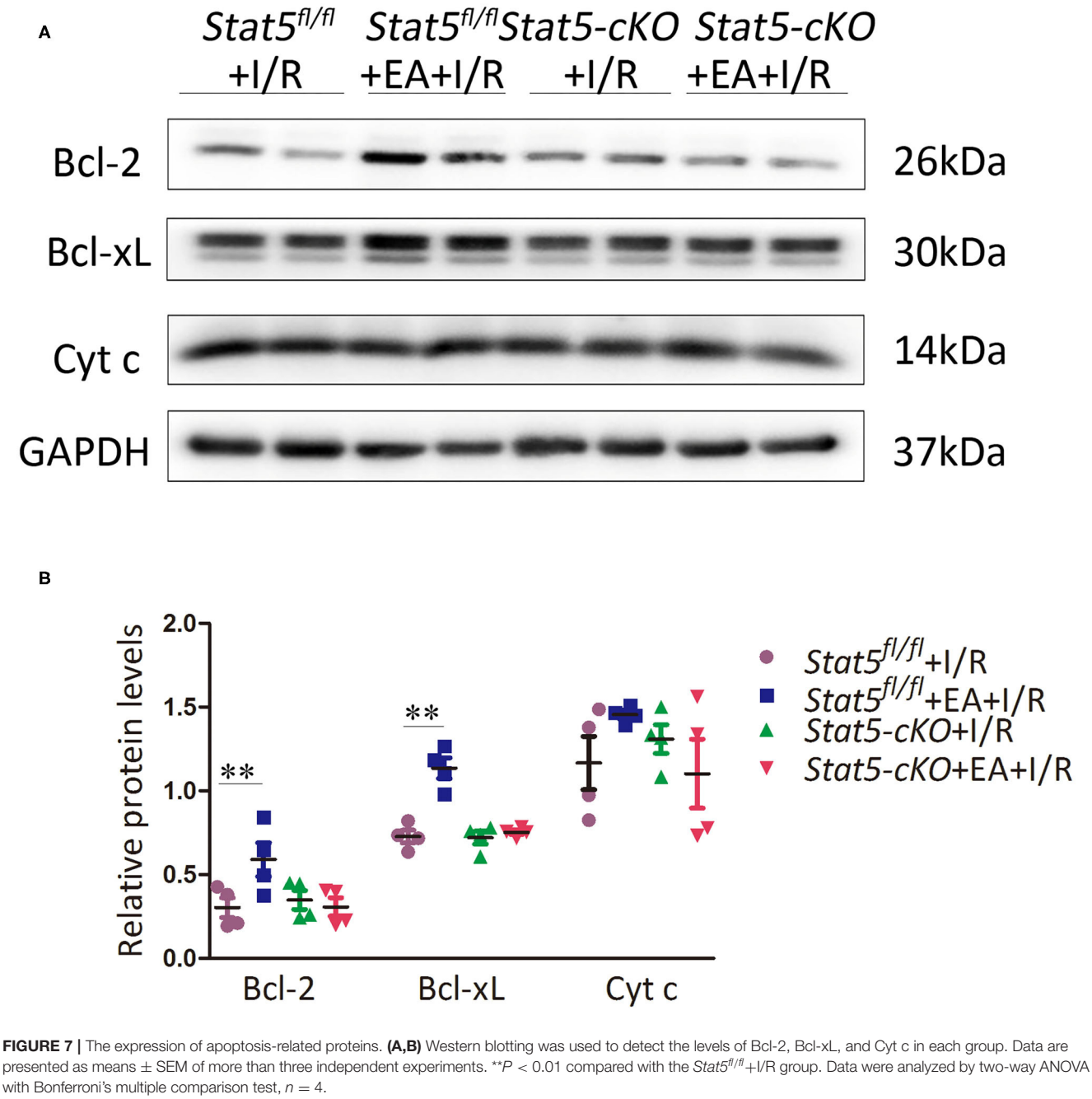

FIGURE 7 | The expression of apoptosis-related proteins. **(A,B)** Western blotting was used to detect the levels of Bcl-2, Bcl-xL, and Cyt c in each group. Data are presented as means ± SEM of more than three independent experiments. $^{**}P < 0.01$ compared with the *Stat5*$^{fl/fl}$+I/R group. Data were analyzed by two-way ANOVA with Bonferroni's multiple comparison test, $n = 4$.

proteins in myocardial I/R injury have not been investigated. Studies have indicated that their roles in cardioprotection may be species-specific (27, 32, 33, 79).

Interestingly, in our study, the level of p-STAT3 was significantly increased in the *Stat5*-cKO+EA+I/R group compared with that in the *Stat5*$^{fl/fl}$+EA+I/R group (**Figure 4**), suggesting that EAP activates STAT3, and that this contributed to the protective effect of EAP against myocardium I/R injury in *Stat5*-cKO mice. Furthermore, EAP increased the mRNA expression levels of *gp130* and *Il6* only in *Stat5*-cKO mice (**Figure 4B**), supporting that IL-6/gp130/STAT3 signaling may be activated to compensate for the loss of *Stat5* following myocardial I/R injury.

Growing evidence has demonstrated the protective role of STAT3 in the heart (30, 32, 79, 91–93). STAT3 helps mitigate cardiac I/R injury by reducing apoptosis or increasing antiapoptotic signaling, upregulating the expression of cardioprotective proteins, decreasing ROS generation, and inhibiting autophagy (92). In addition, the activation of STAT3 is known to enhance mitochondrial function by regulating the transcription of genes encoding proteins such as Bcl-2, Bcl-xL, and VEGF (30, 79, 80, 91). Consistent with these

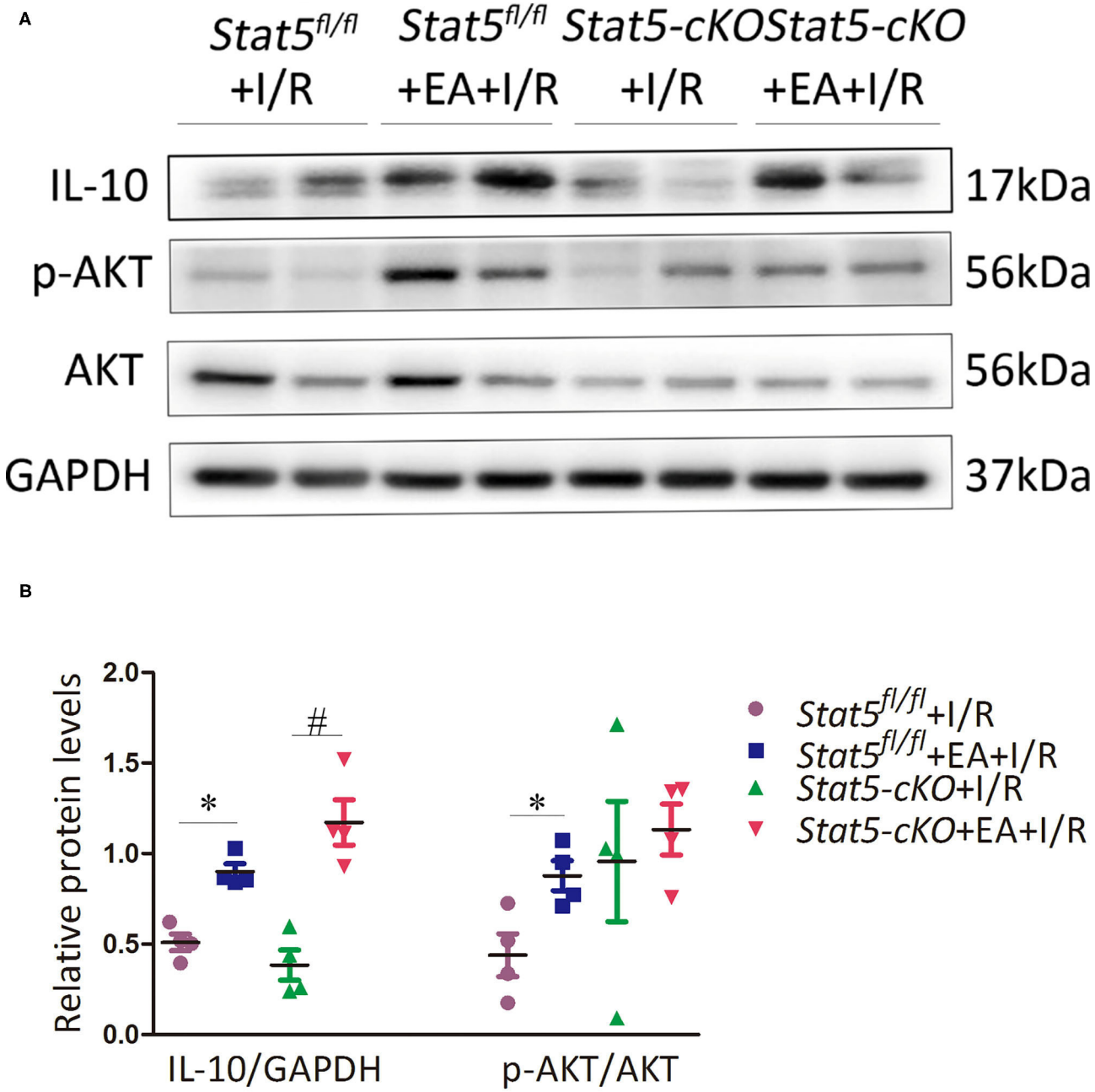

FIGURE 8 | The expression of survival signaling-related proteins. **(A,B)** Western blotting was used to detect the levels of IL-10, p-AKT, and AKT in each group. Data are presented as means ± SEM of more than three independent experiments. $^{*}P < 0.05$ compared with the *Stat5*$^{fl/fl}$+I/R group; $^{\#}P < 0.05$ compared with the *Stat5*-cKO+I/R group. Data were analyzed by two-way ANOVA with Bonferroni's multiple comparison test, $n = 4$.

observations, we found that EAP promoted the expression of Bcl-2, Bcl-xL, and p-AKT in *Stat5*$^{fl/fl}$+I/R mice, which was associated with the activation of IL-6/STAT3 signaling. Notably, IL-10 protein expression was increased in both the *Stat5*$^{fl/fl}$ and the *Stat5*-cKO mice when EAP was applied followed by I/R injury. IL-10 is an important anti-inflammatory cytokine that can be produced by most cell types, and can affect the growth and differentiation of various hematopoietic cells, as well as increase cell proliferation, angiogenesis, and immune evasion (94, 95). We have previously shown that RIPC can activate the expression of IL-10, p-AKT, Bcl-2, and Bcl-xL, thereby protecting the myocardium (37). Recently, Takahashi et al. (96) showed that IL-22, a member of the IL-10 cytokine family, can activate the myocardial STAT3 signaling pathway and protect against myocardial I/R injury in mice. Other studies have also shown that members of the IL-6 and IL-10 families of

cytokines can activate the JAK/STAT3 signaling pathway and induce the transcription of genes involved in cell survival and proliferation (92, 97). In this study, EAP altered the expression of the *Mmp3*, *Ubb*, and *Myh7* genes, which are closely related to the STAT3 pathway, in *Stat5*-cKO mice with myocardial I/R injury (**Table 1B**). This suggested that STAT3 may have played a vital cardioprotective role by controlling the expression of these genes, and may also have activated the functions of macrophages and mononuclear phagocytes in its role as a transcriptional regulator of anti-inflammatory-related genes (98–101). Angiogenesis is an indicator of cardioprotection and STAT3 can promote the expression of VEGF, a key angiogenic factor (102, 103). In our study, the expression of VEGFA did not differ among the four groups (**Supplementary Figure 1**), suggesting that the activation of STAT3 by EAP may not be enough to promote angiogenesis in *Stat5*-cKO mice. Further investigation is needed to clarify this observation.

This study had several limitations. We found that, with EAP, IL-6/gp130/STAT3 signaling was activated in the absence of *Stat5* following I/R injury; however, we did not determine the levels of the associated proteins. Additionally, we did not assess the influence of EAP on mitochondrial function, instead of presenting the apoptotic data alone. The sample size in some experiments was also too small to draw firm conclusions owing to the limited border zone of the heart tissue, even though we pooled 2–3 samples for mRNA extraction to ensure biological duplication. Finally, whole western blots should be presented and not the cut-off pieces.

In summary, in the present study, we demonstrated that EAP can protect against myocardial I/R injury by reducing the myocardial infarct area and activating antiapoptotic, anti-inflammatory, and survival signaling pathways. Although STAT5 is involved in this process, the protective effect of EAP is not STAT5-dependent. STAT3 may compensate for the function of STAT5 in the absence of the *Stat5* gene. Our results suggested that EAP can mimic RIPC but is more effective at protecting the heart against I/R injury.

DATA AVAILABILITY STATEMENT

The original RNA-seq data in our study are publicly available. This data can be found at the sequence read archive (SRA) in NCBI under the accession number PRJNA738960.

ETHICS STATEMENT

The animal study was reviewed and approved by the Institute for Animal Care and Use Committee at Nanjing University of Chinese Medicine.

AUTHOR CONTRIBUTIONS

B-MZ and X-YJ conceived and supervised experiments. H-HG, X-YJ, and B-MZ wrote and edited the manuscript. HC and H-HG performed the experiments and analyzed the data. H-XX carried out the bioinformatic analyses for RNA-seq. 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/fmed.2021.649654/full#supplementary-material

Supplementary Figure 1 | The protein expression of VEGFA. Western blotting analysis was used to determine the level of VEGFA in each group. Data are presented as means ± SEM. No differences were found among the four groups. Data were analyzed by two-way ANOVA with Bonferroni's multiple comparison test, $n = 4$.

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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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Autoantibody-Specific Signalling in Pemphigus

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Pemphigus is a severe autoimmune disease impairing barrier functions of epidermis and mucosa. Autoantibodies primarily target the desmosomal adhesion molecules desmoglein (Dsg) 1 and Dsg 3 and induce loss of desmosomal adhesion. Strikingly, autoantibody profiles in pemphigus correlate with clinical phenotypes. Mucosal-dominant pemphigus vulgaris (PV) is characterised by autoantibodies (PV-IgG) against Dsg3 whereas epidermal blistering in PV and pemphigus foliaceus (PF) is associated with autoantibodies against Dsg1. Therapy in pemphigus is evolving towards specific suppression of autoantibody formation and autoantibody depletion. Nevertheless, during the acute phase and relapses of the disease additional treatment options to stabilise desmosomes and thereby rescue keratinocyte adhesion would be beneficial. Therefore, the mechanisms by which autoantibodies interfere with adhesion of desmosomes need to be characterised in detail. Besides direct inhibition of Dsg adhesion, autoantibodies engage signalling pathways interfering with different steps of desmosome turn-over. With this respect, recent data indicate that autoantibodies induce separate signalling responses in keratinocytes *via* specific signalling complexes organised by Dsg1 and Dsg3 which transfer the signal of autoantibody binding into the cell. This hypothesis may also explain the different clinical pemphigus phenotypes.

Keywords: adhesion, pemphigus, signalling, autoantibodies, autoimmune blistering disease

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INTRODUCTION

Pemphigus is a severe autoimmune blistering skin disease disrupting desmosomes and thereby affecting the epidermis of the skin and the mucosa (1, 2). Desmosomes are important epithelial cell-cell contacts providing high mechanical stability (3–5). Disruption of desmosomes causes the cells to detach from each other, leading to acantholysis. The desmosomal transmembrane cadherins comprise desmogleins (Dsg) 1–4 and desmocollins (Dsc) 1–3 (3). They are connected to the plaque proteins plakoglobin (PG), plakophilin (PKP) 1–3, and desmoplakin (DP). The plaque anchors the desmosomes to the keratin filament cytoskeleton (6–11). The composition of desmosomes varies within tissues and different layers of the epidermis which is relevant since Dsg isoforms differ in their function to regulate signalling pathways in pemphigus. Dsg2, which is ubiquitously expressed throughout other epithelia, can only be found in the basal layer of neonatal epidermis but is absent in adult epidermis outside of hair follicles (12–14). In contrast, Dsg1 and Dsc1 expression increase whereas the amount of Dsg3 and Dsc3 decrease towards superficial epidermal layers with Dsg3 being absent in the stratum granulosum. Dsg4 on the other hand is missing from basal cells, described to be only found in the granular layer and hair follicles (13). Starting from the granular layer, corneodesmosine starts additionally linking cadherins together in the extracellular space (13). This shift in composition marks the stepwise maturation of the adaptable desmosomes towards stable corneodesmosomes found in the cornified layer. In mucosal epithelium, the situation is

slightly different. Dsg2 is found in the basal layers only (15). Dsg1 is less predominant and particularly absent in the basal layer of mucosa whereas Dsg3 is present in all layers (15). Dsg4 can also be found in all layers above the basal layer. In contrast, Dsc1 is not present in oral mucosa while Dsc2 and 3 are present in all layers (3). The expression patterns of desmosomal cadherins in the mucosa are thus more homogeneous compared to epidermis. In neonatal mouse skin, it was shown, that the more ubiquitous distribution of Dsg3 without a distinct enrichtment of Dsg1 in the upper epidermal layers (16).

All pemphigus variants are rather rare and here we focus on the two main variants of the disease pemphigus vulgaris (PV) and pemphigus foliaceus (PF). Incidence and prevalence of PV vary profoundly in different geographical regions as well as ethnicities. In Germany, the prevalence for PV in 2014 was 94.8 per one million population (pmp) (17). It was shown that some genetic variations particularly of the HLA class II (18–24) but also non-HLA genes (23, 25–37), including *DSG3* (38) play a role. Especially, different expression of the transcription factor ST18, which alters signalling pathways including ERK and is associated with apoptosis, was found tocorrelate with increased prevalence in of PV (22, 32, 39, 40). Of course, environmental risk factors might be of importance as well.

For PF, the prevalence was 10.01 pmp (17), with an estimated incidence rate of <1 pmp in the USA and Europe. PF can manifest sporadically, usually at a middle age. It was shown, that genetic-HLA (41–45) and non-HLA markers (28, 46) are relevant as well. However, there are also more frequent endemic PF variants, e.g., in some subtropical areas of Brazil with incidence rates as high as 3–5% and an onset often at a relatively young age (47–52). Other regions such as Tunisia (53) or Colombia (54) show similar variants.

In most cases, PV initially shows mucosal erosions sometimes spreading to the oesophagus, the airways, the anogenital mucosa (2, 55) and in rare cases even the conjunctiva (56) (**Figure 1**). In about half of the cases after mucosal erosions additional epidermal lesions develop (57). In contrast, PF affects the more superficial layers of the epidermis only and is less severe (2, 58, 59), however a spread to deeper layers as well as the mucosa and thus progression to PV is possible (60).

For diagnosis of pemphigus, the specific histology with separation at the suprabasal layer for PV and at the granular layer for PF is important (3, 61) (**Figure 1**). The second specific diagnostic marker are the circulating auto-antibodies against Dsg1 and Dsg3 and in some cases against Dsc3 (57, 61–66), Dsc1 (65), or Dsc2 (66, 67) as well as the accumulation of auto-antibodies in perilesional skin (55, 57). Auto-antibody titers often correlate with disease severity (57, 61, 68, 69).

Pemphigus is a very severe disease with a mortality of around 71% if untreated. Secondary infections due to the disruption of the skin barrier are the most severe problem (58, 70). All treatment strategies aim to reduce the levels of circulating pathogenic autoantibodies. With the introduction of systemic corticosteroid therapy for unspecific immunosuppression, the mortality rate was significantly improved to about 21% (71). However, especially under long-time therapy, these medications cause many side effects including increased infection tendency,

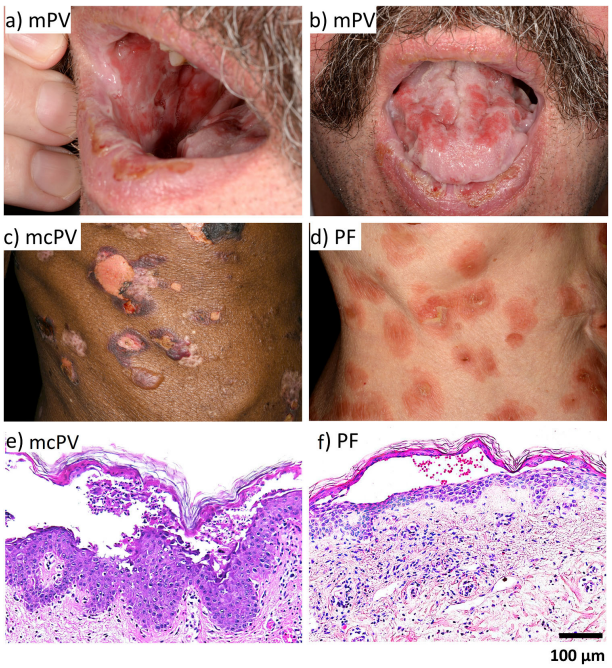

FIGURE 1 | The clinical phenotype and histology of PV and PF. **(a)** mPV. **(b)** mPV. **(c)** mcPV. **(d)** PF. **(e)** mcPV. **(f)** PF.

osteoporosis, low blood sugar, weight gain arterial hypertonia, cataracts, and glaucoma. These side effects can be reduced or delayed by combination therapy with other immune-suppressive agents or antibodies such as Rituximab, which depletes pathogenic B cells, to reduce the steroid dose (72–75). Current combination therapies reduce the mortality to about 5–10% with death mostly resulting from adverse drug side effects (76). These agents usually show less severe but nonetheless considerable side effects including liver toxicity, anaemia, neutropenia, increased infection risk, cold-like symptoms, increased thirst and urination or in some rare worst cases neurological symptoms reaching from sight loss to impaired movement or speech. Patients might also develop an insensitivity to many of these agents (73, 77).

Novel therapy options are for example intravenous injection of high doses of IgG of healthy donors (78, 79), competitively blocking Fc-receptors of inflammatory cells, reducing B-cell response and inducing autoantibody catabolism (80). Another successful concept is plasmapheresis, where the patient's pathogenic autoantibodies are removed (76). In recent years, plasmapheresis is replaced by immunoapheresis, depleting the patient's own autoantibodies by protein A immunoadsorbtion and thus causing less side-effects (81, 82). However, all these therapies have their own drawbacks, including the high costs (83), availability of donor IgG (84) and the need for regular long treatment sessions. Currently the treatment with corticosteroids in combination with immune suppressive agents and Rituximab is still the standard method (72, 85, 86). Furthermore, it is known that not all patients respond to these therapy concepts.

There are some experimental therapy approaches including humanised anti-CD20 antibodies other than Rituximab, designed

to be more specific, more potent and/or less immunogenic (87–89). Antibodies directed against targets other than CD20 are also in development (90–92). A similar approach is targeting B-cell signalling and crosstalk with specific inhibitors (93, 94). Furthermore, IgE signalling *via* IL-4 on Th2 cells which are involved in pemphigus pathology are a novel target (95–97). Targeting Abl-thyrosine kinase-mediated extravasation of autoantibodies into the skin seems to be a promising approach, too (98–100). Another target impacting the autoantibody function are Fc receptors, which prevent IgG degradation and increase autoantibody halflife (101–103). Another approach is to restore Dsg tolerance (104, 105) by transferring Foxp3-expressing regulatory T cells which were shown to be markedly reduced in pemphigus (95). A different emerging option is the therapy with chimeric antigen receptor T-cells (CAR-T)/chimeric autoantibody T-cells (CAA-T) which target a specific antigen and kill the target cell. They could thus deplete autoreactive B-cells (106–108).

For all therapy approaches to reduce autoantibody formation one limitation is that time is required until patients benefit. Therefore, for the acute phase of disease as well as for patients suffering from relapses additional therapeutic options directly stabilising keratinocyte desmosomal adhesion would fulfil an unmet medical need. Directly interfering with keratinocyte signalling to stabilise desmosomes and to induce desmosome assembly could thus be a very promising therapeutic target.

THE ROLE OF AUTOANTIBODIES

Pathogenic autoantibodies in pemphigus are mostly directed against Dsg1 and Dsg3. Antibodies against other desmosomal and non-desmosomal proteins are also found. However, whilst it was shown that anti-Dsg1 and anti-Dsg3 antibodies are pathogenic, the role of most other antibodies for the pathology of pemphigus is not clear (2, 58, 105–112). One rare exception are the autoantibodie against Dsc isoforms such as Dsc3 which induce a pathology similar to anti-Dsg antibodies (57, 61–67). Nevertheless, it is likely that some of these auto-antibodies may cause additive effects which affect the clinical phenotype (109). To address this possibility, the so-called "multiple hit hypothesis" postulates that for the actual disease to occur the cumulative effects of several of these antibodies against desmosomal and non-desmosomal antigens are needed (110, 111). However, AK23, which is a Dsg3-specific antibody from an active mouse model, and monoclonal autoantibodies targeting Dsg1 isolated from PF patients have been shown to induce pemphigus-typical acantholysis (112, 113). This demonstrates that autoantibodies against Dsg molecules are sufficient to cause pemphigus, at least under experimental conditions.

Pathogenic pemphigus autoantibodies targeting Dsg1 and Dsg3 are mostly directed against EC1 and 2, while antibodies directed for example against EC5 are reported to be non-pathogenic (23, 114). It is theorised that pathogenic pemphigus autoantibodies can evolve from the non-pathogenic antibodies *via* epitope spreading. It is known that non-pathogenic Dsg1 autoantibodies are increased in unaffected individuals of high incidence endemic populations (23, 45, 115, 116). In endemic PF, the subtype of IgG usually was found to be IgG4 whereas antibodies in unaffected individuals were almost exclusively IgG2 (117). However, non-pathogenic and pathogenic antibodies often share the same light and heavy chains (114), providing credence to this theory (23, 95, 114). Epitope spreading from Dsg3 to Dsg1 is described in the relatively frequent transformation from mucosal-dominant (anti-Dsg3-IgG only) to mucocutaneous PV (anti-Dsg1- and anti-Dsg3-IgG). The same is true for a less frequent transformation from PF (anti-Dsg1-IgG only) to PV (anti-Dsg1- and anti-Dsg3-IgG) (58, 70). Similarly, autoantibodies against plaque proteins or Dsc isoforms also support this hyposthesis (95). A further piece of evidence is the observation that the introduction of a single Dsg3-reactive T-cell was sufficient to induce the expression of polyclonal anti-Dsg3 IgG in mice *in vivo* (118).

The importance of these most prevalent pemphigus autoantigens is demonstrated further by models to deplete the expression of the corresponding proteins. It was shown that knockout of Dsg3 in mice alone is sufficient to cause a pemphigus-like phenotype (119–121). Similar results were observed for deletion of Dsc3 (122), the autoantigen of atypical pemphigus (123). Similar results were observed in an active Dsc3 mouse model producing autoantibodies against Dsc3 *in vivo* (124). In contrast, Dsg1 K.O. mice show a complete abrasion of the superficial epidermis comparable to the histology in PF during normal birth and are not viable (125).

PATHOGENESIS INDUCED BY AUTOANTIBODIES TARGETING Dsg1 AND Dsg3 AS REVEALED BY EXPERIMENTAL MODELS

Under experimental conditions, autoantibodies against Dsg1 and Dsg3 have been demonstrated to disturb the amount and localisation of the respective desmosomal cadherins as well as to induce reorganisation of the cytoskeleton. anti-Dsg3-IgGs cause reorganisation and depletion of Dsg3 from the cell surface (126, 127). In the skin of PV patients less Dsg3 compared to healthy individuals was detectable (128). Both PV-IgG (126, 129) and the monoclonal antibody AK23 reduced membranous Dsg3 *in vitro* (130) in human skin *ex vivo* (127) and in neonatal mice *in vivo* (127). Starting at a reduction by about 50%, cell adhesion *in vitro* was significantly reduced (130). For mice injected with PF-IgG a reduction of Dsg1 by about 30% was enough to cause blistering (128). In line with this, blister formation in neonatal mice was inhibited under conditions which blocked the depletion of Dsg3 (127). The internalisation of Dsg3 occurs *via* endocytosis in a complex with PG. Following the internalisation, the proteins are degraded in the lysosome (126).

Despite the well-established importance of Dsg3 depletion in pemphigus pathogenesis it is yet unclear if this is an early or late mechanism (3). Furthermore, it was shown that different Dsg3 fractions exist which behave differently in terms of depletion (129–132). This is important since

localisation of desmosomal cadherins is not limited to the desmosomes (133, 134). Rather, extradesmosomal Dsg molecules (135–137) linked to the actin cytoskeleton (138) are reported as well. It was observed that the extradesmosomal Dsg3 is depleted relatively rapidly following exposure to pemphigus autoantibodies whereas it takes much longer until the Dsg3 integrated in desmosomes is notably impacted by pathogenic IgG (129–131, 139). Since it was observed that acantholysis is actually initiated in the spaces between the desmosomes (140, 141) while keratinocytes stay connected punctually *via* the desmosomes (142) which are disrupted later (143–145), it is hypothesised that the exradesmosomal Dsg3 complexes represent desmosome precursors, which are later integrated into desmosomes (135, 146). This process could possibly be disrupted by pathogenic autoantibodies leading to endocytosis and degradation of these precursors (129, 135, 139). Since desmosomes are dependent on a dynamic equilibrium and are constantly remodelled under high molecular fluctuation (147, 148) this would prevent desmosome formation and cause disassembly of existing desmosomes (135, 149). A different hypothesis proposes a membrane-receptor-like function for non-desmosomal Dsg3 (59, 144, 150) suggesting that extradesmosomal and desmosomal cadherins may have distinct roles.

Together with the disorganisation of the desmosome a retraction of keratin filaments from the cell surface occurs (151–153). During this process, keratin filaments detach from the desmosomes (142, 143, 145, 154) and concentrate around the nucleus (141, 153–156). It was shown that the retraction of keratin filaments starts even before the internalisation of Dsg3 and correlates with the time the cell dissociation starts. Keratin filaments show a clear association with Dsg3 reorganisation at the membrane, preceding endocytosis (153) and turnover after PV-IgG treatment (151). This mechanism is thus proposed to play an important role in pemphigus pathogenesis and especially the time of onset before desmosome disassembly suggest keratin filament retraction to be a primary pathogenic mechanism (153). The actin cytoskeleton was also shown to be affected by PV-IgG which may interfere with desmosome assembly (129, 157).

MECHANISMS CAUSING ACANTHOLYSIS IN PEMPHIGUS

Meanwhile it is widely accepted that the mechanisms underlying loss of cell adhesion in pemphigus comprise both direct inhibition of Dsg interaction by bound autoantibodies and signalling mechanisms regulating keratinocyte adhesion triggered by autoantibody binding (158). In the pioneering studies on pathologic mechanisms direct inhibition of Dsg3 adhesion by steric hindrance was very suggestive to be causal for acantholysis (159) whereas signalling such as PLC-induced Ca^{2+} release (156, 160) was regarded more as secondary bystander effect.

Meanwhile, there are some studies available on steric hindrance of Dsg1 and Dsg3 adhesion, which for technical reasons are mostly carried out under cell-free conditions *in vitro* using bead assays or single-molecule atomic force microscopy (161) but rarely were performed in cultured keratinocytes (**Table 1**). However, studies using *in vivo* models or human organ culture are lacking which makes it hard to decide whether effects of autoantibodies on recombinant extracellular domains of Dsg1 and Dsg3 can be transferred to the situation in intact skin where desmosomes are formed in a complex multi-step process. In contrast, there is ample evidence for the role of signalling pathways in the regulation of desmosomal adhesion in PV from studies not only performed in cultured cells but also using *in vivo* mouse models and *ex-vivo* human epidermis whereas data for PF are still limited (**Table 2**).

DIRECT INHIBITION OF DESMOGLEIN ADHESION VS. CELLULAR SIGNALLING

The cadherins were reported to undergo homo- as well as heterophilic Ca^{2+}-dependent interactions *via* their extracellular domains (3). The outermost extracellular domain EC1 domain seems to be most important for Dsg adhesion (1, 27, 162–164). As discussed above, the pathogenic autoantibodies found in pemphigus mostly bind to this domain (27, 162, 165, 166). At first glance, it appears obvious that the binding of a large protein like an antibody to this region would cause steric hindrance by directly inhibiting the Dsg binding and thus cell adhesion. For Dsg3 it was possible to show the occurrence of such a direct inhibition by PV-IgG *in vitro*, both under cell-free conditions and for adhesion to the cell-surface (151, 167) (**Table 1**). A PV-mouse-model did also show possible indications of direct inhibition of Dsg3 adhesion (165). The finding that the monoclonal anti-Dsg3-antibody AK23, which causes a PV-like phenotype in mice (112), also causes direct inhibition of homophilic Dsg3 interaction under cell-free conditions supports this hypothesis (152, 168). Homophilic Dsc3 and heterophilic Dsc3-Dsg1 were reported to be important for adhesion in keratinocytes as well. Direct inhibition of these interactions greatly reduced the adhesive strength (64). However, another study reported heterophilic interactions between Dsg1 and Dsc1 and Dsg3 and Dsc3 to be the fundamental adhesive unit of desmosomes. In a cell-free assay, mixtures of Dsg1 and Dsc1 or Dsg3 and Dsc3 ectodomains formed large aggregates while Dsg1 or Dsg3 as well as Dsc1 or Dsc3 alone only formed minor to no aggregates. Autoantibodies where effective to directly inhibit binding of Dsg1 to Dsc1 and Dsg3 to Dsc3 beads (169). Direct inhibition has thus to be considered to play a role in PV pathology (150, 167, 170). However, direct inhibition alone is not adequate to cause a complete loss of keratinocyte adhesion. This is highlighted by the fact that impairing cellular signalling but not direct inhibition by incubating the cells at 4 °C did prevent PV-IgG induced loss of cohesion (126). Moreover, inhibition of p38MAPK rescued cell adhesion although direct inhibition of Dsg3 binding by PV-IgG was still detectable which demonstrates that modulation of autoantibody-induced signalling is sufficient to outbalace effects of steric hindrance (137). For PF, the intracellular signalling seems to be even more important since no direct inhibition of Dsg1 interaction was observed in several studies (151, 152, 168, 171).

TABLE 1 | Studies on direct inhibition of Dsg1 and Dsg3 interaction in pemphigus.

PV	***In vitro* (cell-free)**	***In vitro* (cultured cells)**	***In vivo* (mice)**	***Ex vivo (human)***
Dsg1	**No inhibition** (homophilic): AFM – Heupel, JI 2008 – Walter, Sci Rep 2017 (heterophilic Dsc3): AFM – Spindler, JBC 2009 **Inhibition** (heterophilic Dsc1): beads – Ishii, JID 2020 **No inhibition** (homophilic): AFM – Walter, SR 2017	No Data	No Data	No Data
Dsg3	**Inhibition** (homophilic): AFM – Heupel, JI 2008 – Heupel, JBC 2009 – Spindler, JCI 2013 – Walter, SR 2017 **Inhibition** (heterophilic Dsc3): beads – Ishii, JID 2020	**Inhibition** (homo/heterophilic): AFM – Vielmuth, JID 2015	**Inhibition** No Data	No Data
PF	***In vitro* (cell-free)**	***In vitro* (cultured cells)**	***In vivo* (mice)**	***Ex vivo* (human)**
Dsg1	**No inhibition** (homophilic): AFM – Waschke, JCI 2005 **Inhibition** (heterophilic Dsc1): – Ishii, JID 2020 **No inhibition** (homophilic): AFM – Walter, SR 2017	**No inhibition** (homo/heterophilic): beads – Waschke, JCI 2005 **No inhibition** (homo/heterophilic): AFM – Vielmuth, Frontiers Immunol 2018	No Data	No Data

Over time, several signalling pathways have been identified to be essential for the pathology of pemphigus (59, 152, 171–173) (**Table 2**). The focus of current research is to integrate all the different pathways and to define their role in pemphigus pathogenesis. Especially, the chronology of many steps is not clear at present.

THE PATHOLOGY OF PATIENTS' LESIONS IDENTIFIES THE UNDERLYING MECHANISMS INTERFERING WITH DESMOSOME TURN-OVER

Microscopic evaluation of skin lesions in pemphigus revealed that the pathogenic mechanisms involved cannot be simple. Rather, the different clinical phenotypes in PV and PF are defined by characteristic histology and ultrastructural alterations of desmosomes, which only can be explained by severe impairment of desmosome turn-over, have been established as hallmarks of the disease.

In PV, deep epidermal blisters and mucosal erosions typically separate supra-basal keratinocytes in the blister roof from tombstone-like cells of the basal layer in the blister bottom (174, 175) (**Figure 1**). In contrast, superficial erosions in PF tear of granular and apical spinous cell layers. Since Dsg3 is the predominant desmosomal cadherin in oral mucosa whereas Dsg1 is sparsely expressed, it is conceivable why in PF mucosal erosions usually are absent. Similarly, because in the granular layer Dsg1 and Dsc1 are the main desmosomal cadherins expressed, superficial blistering in PF can be explained by the Dsg compensation hypothesis (176). Supporting this, it was reported, that in neonatal mouse skin, the more ubiquitous distribution of Dsg3 without a distinct enrichtment of Dsg1 in the upper epidermal layers provided a protective effect against PF-IgG (16). However, the morphology of epidermal lesions in PV does not reflect the distribution patterns of Dsg1 and Dsg3 which in human skin broadly overlap. Thus, desmoglein compensation cannot explain a sharp supra-basal cleavage plain as found in PV (3, 150). Also, morphologic alterations typical for apoptosis usually are not present on histologic or ultrastructural levels in patients' lessons demonstrating that apoptotic cell death is not a major cause for acantholysis (154, 177, 178).

It has been shown that Dsg3 clustering in the absence of epidermal blistering correlates with the presence of autoantibodies against Dsg3 in patients with mucosal-dominant PV (149, 178, 179). Similarly, inter-desmosomal widening in PV and PF was present in unaffected epidermis suggesting that these events are not sufficient to cause skin blistering. Rather,

TABLE 2 | Studies on signalling pathways in pemphigus.

PV	Activation	Pathogenic *In vitro* (cultured cells)	Pathogenic *In vivo* (mice)	Pathogenic *Ex vivo* (human)
p38 MAPK	– Berkowitz, JBC 2005 – Berkowitz, PNAS 2006 – Chernyavsky, JBC 2007 – Berkowitz, JID 2008 – Lee, JBC 2009 – Marchenko, JBC 2010 – Spindler, JI 2010 – Mao, JBC 2011 – Bektas, JBC 2013 – Spindler JCI 2013 – Mao, JID 2014 – Spindler, JID 2014 – Hariton, ED 2017 – Walter, Sci Rep 2017 – Vielmuth, FI 2018 – Jin, BA 2021	– Berkowitz, JBC 2005 – Chernyavsky, JBC 2007 – Lee, JBC 2009 – Mao, JBC 2011 – Bektas, JBC 2013 – Spindler JCI 2013 – Mao, JID 2014 – Rötzer, JBC 2014 – Spindler, JID 2014 – Walter, Sci Rep 2017 – Vielmuth, FI 2018 – Burmester, BJP 2020	– Berkowitz, PNAS 2006 – Spindler JCI 2013 – Mao, JID 2014	– Saito, PO 2012 – Egu, BJD 2017 – Burmester, BJP 2020
MK2	– Mao, JID 2014 – Egu, BJD 2019	– Mao, JID 2014	– Mao, JID 2014	No data
HSP25/27	– Berkowitz, JBC 2005 – Berkowitz, JID 2008 – Bektas, JBC 2013 – Mao, JID 2014	No data	No data	No data
RhoA	– Waschke, JCB 2006 – Jin, BA 2021	– Waschke, JCB 2006 – Spindler, AJP 2007 – Gliem, AJP 2010 – Jin, BA 2021	No data	– Waschke, JCB 2006
Adducin	– Rötzer, JBC 2014	No data	No data	No data
Ca^{2+}	– Esaki, JID 1995 – Seishima, JID 1995 – Rötzer, JBC 2014 – Walter, Sci Rep 2017 – Schmitt, BJD 2021	– Esaki, JID 1995 – Arredondo, AJP 2005 – Rötzer, JBC 2014 – Walter, FI 2019 – Schmitt, BJD 2021	No data	– Schmitt, BJD 2021
PI4K	No data	– Schmitt, BJD 2021	No data	No data
PLC	– Seishima, JID 1995 – Schmitt, BJD 2021	– Esaki, JID 1995 – Schmitt, BJD 2021	– Sanchez-Carpintero, BJD 2004	– Schmitt, BJD 2021
IP3R	No Data	– Schmitt, BJD 2021	No data	– Schmitt, BJD 2021
CRAC	No Data	– Schmitt, BJD 2021	No data	No data
PKC	– Osada, JID 1997 – Kitajima, JID 1999 – Rötzer, JBC 2014	– Kitajima, JID 1999 – Cirillo, J Cell P 2010 – Spindler, AJP 2011 – Walter, Sci Rep 2017	– Sanchez-Carpintero, BJD 2004 – Spindler, AJP 2011	– Egu, Frontiers 2019
cAMP/PKA	– Spindler, JI 2010 – Walter, SR 2017	– Spindler, JI 2010	– Spindler, JI 2010	– Meier, 2020 FI
Src	– Chernyavsky, JBC 2007 – Pretel, ED 2009 – Marchenko, JBC 2010 – Gil, ED 2012 – Tsang, JP 2012 – Walter, Sci Rep 2017 – Kugelmann, FI 2019	– Chernyavsky, JBC 2007 – Cirillo, AI 2014 – Walter, SR 2017 – Kugelmann, FI 2019	– Pretel, ED 2009 – Gil, ED2012	Not protective
ADAM10	– Ivars, BJD 2020	– Ivars, BJD 2020	No data	No data
MMPs	– Grando, JDS 1992 – Cirillo, JCP 2007	No data	No data	No data
EGFR	– Frusić-Zlotkin, AI 2006 – Chernyavsky, JBC 2007 – Pretel, ED 2009 – Marchenko, JBC 2010 – Bektas, JBC 2013	– Frusić-Zlotkin, AI 2006 – Bektas, JBC 2013 – Sayar, ED 2014 – Walter, FI 2019	– Pretel, ED 2009	No data

(Continued)

TABLE 2 | Continued

PV	Activation	Pathogenic *In vitro* (cultured cells)	Pathogenic *In vivo* (mice)	Pathogenic *Ex vivo* (human)
	– Sayar, ED 2014 – Walter, FI 2019			
PI3K	No data	– Burmester, BJP 2020	No data	– Burmester, BJP 2020
PDK1	No data	– Burmester, BJP 2020	No data	No data
mTor	– Pretel, ED 2009 – Gil, ED2012	No data	– Pretel, ED 2009	No data
FAK	– Gil, ED2012	No data	– Gil, ED2012	No data
nNOS	No data	No data	– Espana, ED 2013	No data
Casp3	– Arredondo, AJM 2005 – Frusić-Zlotkin, AI 2006 – Dusek, JBC 2006 – Lee, JBC 2009 – Marchenko, JBC 2010 – Pacheco-Tovar, AD 2011 – Gil, ED2012 – Luyet, PO 2015 – Hariton, ED 2017	– Arredondo, AJM 2005 – Frusić-Zlotkin, AI 2006 – Marchenko, JBC 2010 – Pacheco-Tovar, AD 2011 – Luyet, PO 2015 – Schmitt, AJPhysiol 2009	– Pretel, ED 2009 – Hariton, ED 2017	No data
Casp8	– Puviani, JID 2003 – Arredondo, AJM 2005 – Marchenko, JBC 2010 – Lotti, FI 2018 – Sanath, JOMP 2018	– Puviani, JID 2003 – Arredondo, AJM 2005 – Marchenko, JBC 2010	– Pretel, ED 2009	No data
Casp9	– Marchenko, JBC 2010 – Gil, ED2012	– Marchenko, JBC 2010	– Pretel, ED 2009	No data
c-Myc	– Williamson, EMBOJ 2006 – Williamson, JID 2007	No data	No data	No data
JNK	– Marchenko, JBC 2010	No data	No data	No data
c-Jun	– Frusić-Zlotkin, AI 2006	No data	No data	No data
PG	No data	– Spindler, JID 2014	No data	No data
ERK/MEK	– Frusić-Zlotkin, AI 2006 – Bektas, JBC 2013 – Rötzer, JBC 2014 – Walter, Sci Rep 2017 – Radeva, Frontiers 2019 – Walter, FI 2019	– Frusić-Zlotkin, AI 2006 – Walter, Sci Rep 2017 – Radeva, Frontiers 2019 – Burmester, BJP 2020	No data	– Egu, FI 2019 – Burmester, BJP 2020
Calmodulin	No data	No data	– Sanchez-Carpintero, BJD 2004	No data
Calpain	– Arredondo, AJM 2005	No data	No data	No data
CytC	– Marchenko, JBC 2010		No data	No data
CETP	No data	– Burmester, BJP 2020	No data	No data
FAS	– Puviani, JID 2003 – Wang, Appt 2004 – Arredondo, AJM 2005 – Lotti, FI 2018	– Puviani, JID 2003 – Wang, Appt 2004 – Marchenko, JBC 2010 – Lotti, FI 2018	No data	No data
FLIP	– Arredondo, AJM 2005	No data	No data	No data
PLK1	No data	– Burmester, BJP 2020	No data	No data
TrkA	No data	– Burmester, BJP 2020	No data	– Burmester, BJP 2020
VEGFR2	No data	– Burmester, BJP 2020	No data	– Burmester, BJP 2020
PF	**Activation**	**Pathogenic *In vitro* (cultured cells)**	**Pathogenic *In vivo* (mice)**	**Pathogenic *Ex vivo* (human)**
p38 MAPK	– Berkowitz, JID 2008 – Lee, JBC 2009 – Walter, Sci Rep 2017 – Vielmuth, FI 2018	– Walter, Sci Rep 2017 – Yoshida, JDS 2017 – Vielmuth, FI 2018	– Berkowitz, AJP 2008 – Lee, JBC 2009	– Yoshida, JDS 2017
HSP25/27	– Berkowitz, AJP 2008 – Berkowitz, JID 2008	No data	No data	No data

(Continued)

TABLE 2 | Continued

PF	Activation	Pathogenic *In vitro* (cultured cells)	Pathogenic *In vivo* (mice)	Pathogenic *Ex vivo* (human)
RhoA	– Waschke, JCB 2006	– Waschke, JCB 2006 – Spindler, AJP 2007	No data	– Waschke, JCB 2006
Src	– Walter, Sci Rep 2017	– Walter, Sci Rep 2017	No data	No data
c-Myc	– Williamson, EMBOJ 2006 – Williamson, JID 2007	No data	No data	No data
ERK/MEK	– Walter, Sci Rep 2017 – Walter, FI 2019	– Walter, Sci Rep 2017	No data	No data
EGFR	– Sayar, ED 2014 – Walter, FI 2019	– Sayar, ED 2014 – Walter, FI 2019	– Pretel, ED 2009	No data
Casp6	– Li, JI 2009	No data	No data	No data
Ca^{2+}	– Seishima, JID 1995 – Walter, Sci Rep 2017 – Walter, FI 2019 – Schmitt, BJD 2021	– Walter, FI 2019 – Schmitt, BJD 2021	No data	No data
PLC	– Seishima, JID 1995	No data	No data	No data
PKC	No data	– Walter, Sci Rep 2017	No data	No data

epidermal blistering occurred when autoantibodies against Dsg1 were present in PV and PF, the number of desmosomes was reduced, desmosomes were smaller in size and keratin filament retraction from desmosomal plaques was identified by electron microscopy (145, 178, 180). Split desmosomes with partial or complete separation of the two desmosomal plaques from neighbouring cells and double-membrane structures, reflecting engulfment of membrane domains, were detected also. Thus, the best strategy to evaluate the relevance of the different mechanisms causing pemphigus pathology is to correlate mechanisms with the morphologic hallmarks of the disease. Direct inhibition of desmoglein binding by steric hindrance through bound autoantibodies would cause splitting of desmosomes but no alterations of desmosome ultrastructure. Indeed, mechanical shear has been demonstrated to cause split desmosomes (181) which therefore may be caused by steric hindrance. Since split desmosomes mostly were found to be smaller and displayed altered insertion of keratin filaments (15, 178, 181, 182), splitting of desmosomes most likely is a consequence of altered desmosome composition. Moreover, most desmosomes in *ex vivo* human skin models of pemphigus are not split but rather smaller and characterised by less opaque plaques, less dense desmoglea in the desmosomal intercellular space and shorter keratin filaments attached (15, 182). Taken together, the ultrastructural alterations can be explained only by impaired desmosome turn-over (178, 183).

Therefore, the major goal is to decipher which mechanisms shown to be induced by autoantibodies interfere with the distinct phases of desmosome assembly and dissasembly. The different steps of desmosome assembly have been characterised in detail (184). First, Dsg and Dsc molecules together with PG are transported to cell borders with preformed adherens junctions along microtubules and *via* kinesin. At the membrane, desmosomal cadherins associate with lipid rafts *via* Flotillin-1 and 2 which are required for proper desmosome assembly (185, 186). Here, intermediate junctions composed of Dsgs and adherens junction components including E-cadherin, PG and β-catenin are assembled in Src-dependent manner (138, 187, 188). In a next step, Dsg molecules are transferred to DP coupled to keratin filaments (9, 189). Actin binding proteins such as adducing, which is regulated *via* RhoA/Rho kinase, and cortactin are important for directed targeting of Dsg molecules during desmosome assembly (190–193). The assembly of Dsgs with DP is controlled by PKP3 in a Rap1-dependent manner (194), the latter of which is a GTPase activated by cAMP and EPAC1. Inside of desmosomes, Dsg isoforms including Dsg1 and Dsg3 in a Ca^{2+}-dependent and most likely homo- and heterophilic manner interact with Dsc1 and Dsc3 *via* their extracellular domains (64, 168, 169, 171, 188, 195).

HYPERADHESION AS A STATE OF INCREASED DESMOSOME STABILITY

Finally, the desmosomal components are locked inside of desmosomes by signalling mechanisms which control the anchorage of DP to keratin filaments and thereby induce a hyperadhesive state (196). This indicates that once a desmosome is established it is remarkably stable but the protein exchange of desmosomal components is regulated on the molecular level resulting in low half-life of desmosomal components (147, 196, 197). This paradigm allows desmosome disassembly when the net exchange by molecules diffusing out of desmosomes outbalances the assembly process as described above in processes like wound healing and migration (198–200). Also, this explains why uptake of entire desmosomes in double-membrane structures as observed under pathologic conditions in pemphigus *ex vivo* models is rarely observed in intact epidermis (182).

Interestingly, under hyperadhesive conditions the binding properties of desmogleins change dramatically. For instance, in hyperdahesive keratinocytes oligomerization of Dsg3 but not Dsg1 is enhanced and the Ca^{2+}-dependency of desmosome integrity is abolished (196, 198, 201–203). Rather, if trans-interaction between Dsg3 molecules was maintained desmosomes were preserved under Ca^{2+}-depletion although the ordered array of the Dsg3 extracellular domains in desmosomes was lost (196). This indicates that Ca^{2+}-dependency of Dsg3 interaction may get lost when adhesion molecules and plaque proteins in desmosomes are locked by cytoskeletal anchorage. Moreover, since it was shown that Dsg1 interaction can be rescued after re-substitution of Ca^{2+} whereas Dsg3 cadherin order was not re-established (171, 196, 204), it is also possible that the roles of Dsg1 and Dsg3 in hyperadhesion are different. This can be concluded from the observation that in intact human epidermis, in which the majority of desmosomes is assumed to be hyperadhesive (198, 201, 202, 205), immunostaining of Dsg1 but not of Dsg3 was drastically altered by Ca^{2+} depletion indicating that Dsg3 in the epidermis indeed loses its Ca^{2+} dependency (206). In line with this, the molecular binding strength of Dsg3 but not of Dsg1 was increased when keratinocytes were hyperadhesive.

As the underlying molecular mechanism regulating hyperadhesion, PKC-mediated phosphorylation of DP under control of PKP1 has been shown to result in decreased cytoskeletal anchorage and thus to revert the hyperadhesive state in order to allow desmosome reorganisation (196, 197, 199–201, 207, 208). To allow this adaption, PKC was shown to be sequestered to desmosomes *via* the adaptor-protein RACK1 in DP-dependent manner and is regulated by keratins (170, 208, 209). However, since in cultured cardiomyocytes and cardiac slice cultures besides inhibition of PKC a hyperadhesive state can also be induced by PKA activation and p38MAPK inhibition, it is likely that other signalling mechanisms involved in the assembly of the desmosomal plaque and its anchorage to keratin filaments are also important for hyperadhesion (210–212). Other targets besides DP appear to be relevant as well because hyperadhesion can be abolished when expression of desmosomal components such as Dsg3 or PKP1 and PKP3 is disturbed (203, 213), which is also similar to cardiomyocytes where depletion of Dsg2, PG, or PKP2 abrogated hyperadhesion (214).

An open question, which also has relevance to identify the central molecular mechanisms impairing desmosome turn-over in pemphigus remains: whether the hyper-adhesive state can be recognised on the level of desmosome ultrastructure. It has been proposed that a dense midline in the desmosome intercellular space where extracellular domains of neighbouring cells trans-interact would be a hallmark of hyperadhesion (198). Indeed, in intact epidermis most desmosomes exhibit dense midlines which is not the case in the vicinity to epidermal blisters or where the desmosome ultrastructure is altered in *ex vivo* human pemphigus skin models (182, 215). However, in cultured keratinocytes under hyperadhesive conditions desmosomes were lacking midlines completely (196). This indicates that the hyperadhesive state cannot be concluded for a specific desmosome from its ultrastructure in cultured cells. On the other hand it can be assumed that in the epidermis desmosomes with altered morphology and lacking midlines most likely are not hyperadhesive and thus PKC is likely to participate in pemphigus pathology by reverting hyperadhesion of desmosomes.

AUTOANTIBODIES AGAINST Dsg1 AND Dsg3 INDUCE SPECIFIC SIGNALLING PATHWAYS IN PEMPHIGUS

As summarised above a vast set of signalling pathways has been associated with pemphigus pathogenesis (**Figures 2–5** and **Table 2**). Before we try to allocate the different mechanisms to the steps of desmosome assembly and disassembly, we introduce the concept that the signalling pathways activated by a specific autoantibody are defined by the signalling complexes organised by Dsg1 and Dsg3. It was an important observation that signalling responses in keratinocytes are not a consequence of loss of cell adhesion but rather signalling molecules are associated with Dsg isoforms. This was shown first for Dsg3, which was found to form a complex with activated p38MAPK, a process enhanced by pemphigus autoantibodies (216). This adhesion receptor was regulated in adhesion-dependent manner because reduced Dsg3 adhesion by specific autoantibodies or peptides or tryptophan caused p38MAPK activation whereas stabilisation of Dsg3 binding by cross-linking peptides reduced p38MAPK activity (216, 217). This process is regulated by PG because depletion of PG also caused p38MAPK activation. In contrast, depletion of DP or PKP1 and PKP3, which also cause a profound loss of keratinocyte adhesion, did not enhance p38MAPK activity (203, 218, 219).

The signalling complexes organised by Dsg molecules appear to be both isoform- and cell type-specific because Dsg2 in contrast to Dsg3 and Dsg1 does not associate with p38MAPK (14, 220, 221). Nevertheless, in enterocytes Dsg2 regulates p38MAPK activity (222). In contrast, Dsg2 has been shown to bind and to control EGFR in enterocytes in fashion similar to Dsg3 in keratinocytes (187, 223).

All of this led us to propose that Dsg molecules form cell-type specific signalling complexes which serve as adhesion-dependent signalling receptors and regulate complex functions such as keratinocyte migration and wound healing (59, 224). Most recently, we observed that Dsg1 also forms signalling complexes which are at least in part different to Dsg3. The majority of signalling molecules involved in pemphigus pathogenesis analysed so far including p38MAPK, PLC, and PKC were associated with both Dsg1 and Dsg3. However, PI4K acting upstream of the PLC/Ca^{2+}/PKC pathway was found exclusively to interact with Dsg1 (220). The fact that p38MAPK binds to both Dsg1 and Dsg3 is compatible with the observation that blister formation *in vivo* caused by PV-IgG and PF-IgG is blocked by inhibition of p38MAPK (225, 226) and that both PV-IgG and PF-IgG reduced activity of RhoA in p38MAPK-dependent manner (127, 227).

Moreover, these findings are in line with the observation that autoantibody fractions including antibodies targeting Dsg1 in

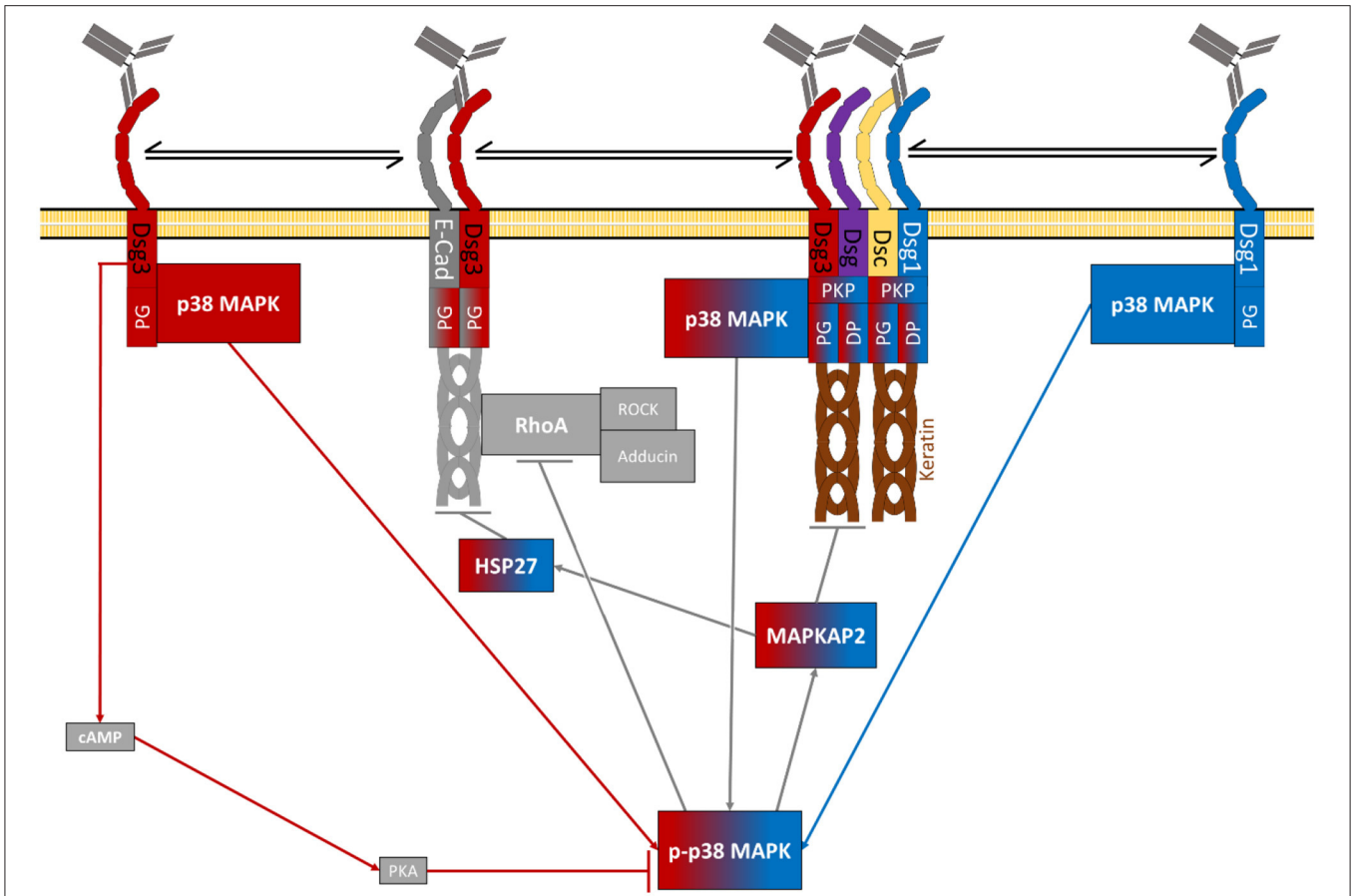

FIGURE 2 | Schematic depiction of p38MAPK-dependent signalling in pemphigus (grey RhoA and cAMP steps represent potential rescue pathways).

PV and PF induce a different signalling response compared to antibodies against Dsg3. Most striking, PLC activation and Ca^{2+} influx was found only when Dsg1 autoantibodies were present in PV-IgG and PF-IgG fractions but not induced by mucosal-dominant PV-IgG or AK23 (152, 220, 228). Similarly, ERK activation was detectable after incubation with PF-IgG and PV-IgG only when Dsg1 antibodies were present and similar to Ca^{2+} influx activation was preserved in Dsg3-deficient keratinocytes. In contrast, EGFR activation was found to be dependent on Src and caused by AK23 and mucosal-dominant PV-IgG and thus in the absence of autoantibodies against Dsg1 (152, 228). Similarly, Src- and ADAM10-dependent EGFR activation has been found in response to PV-IgG including autoantibodies against Dsg1/3 whereas blister formation with additional autoantibodies against Dsc2/3 was ADAM10-independent (66).

For PKC it is not clear whether the different isoforms are regulated *via* Dsg1 or Dsg3. PKC was found to associate with both Dsg1 and Dsg3 and inhibition of PKC ameliorated loss of cell adhesion in response to PF-IgG and PV-IgG independent of the presence of autoantibodies against Dsg1 (152, 220). Also, PKC was found to be involved in Dsg3 depletion *in vivo* (127). On the other hand, at least for Ca^{2+}-dependent PKC isoforms activation *via* autoantibodies against Dsg1 can be assumed (220).

Taken together, the studies on composition of signalling complexes on one hand and evaluation of signalling pathways on the other hand suggest that besides p38MAPK activation autoantibodies against Dsg3 in PV cause SRC-dependent EGFR activation whereas autoantibodies against Dsg1 in PV and PF activate both ERK and the PI4K/PLC/Ca^{2+} pathway. The hypothesis that the signalling pattern orchestrated by Dsg1 and Dsg3 in pemphigus are to some extent different also helps to explain why blister formation in pemphigus requires antibodies against Dsg1. In this line of thoughts, some pathologic effects such as clustering and depletion of Dsg3 molecules in unaffected skin of mucosal-dominant PV patients (149, 179) can be induced by signalling pathways such as p38MAPK, Src/EGFR and PKC. However, clustering of Dsg1 as well as shrinkage and loss of desmosomes which finally cause epidermal blistering (149, 179, 182) require ERK and PLC/Ca^{2+} signalling in addition to p38MAPK. This third line of evidence that the relative contribution of various signalling pathways for skin blistering in pemphigus is different comes from studies in human skin *ex vivo* where inhibition of p38MAPK, ERK, and PLC activity as well as of Ca^{2+} influx were sufficient to abrogate skin blistering whereas inhibition of Src and PKC was not (182, 191, 215, 220, 229).

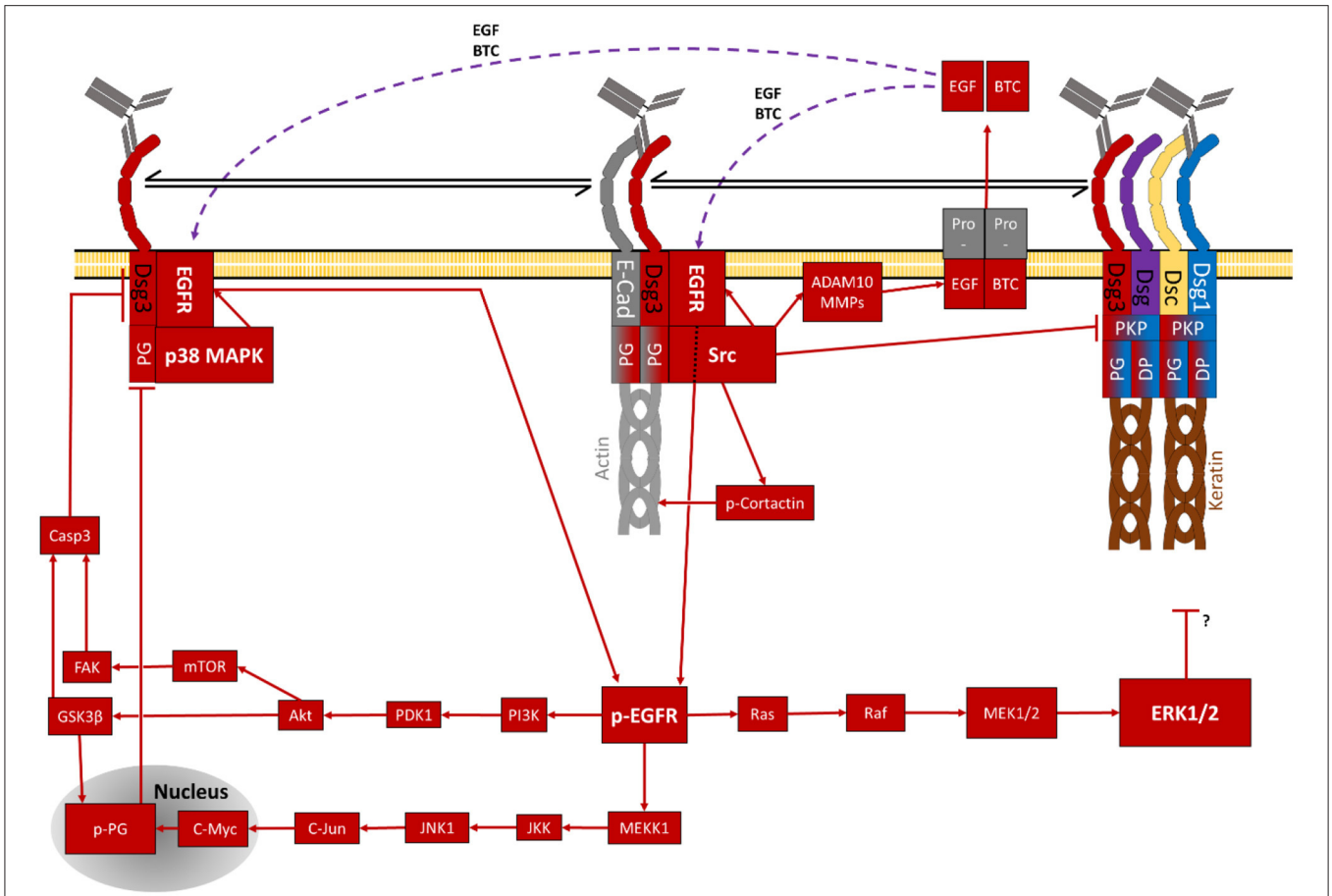

FIGURE 3 | Schematic depiction of EGFR-dependent signalling pathways (purple lines indicate diffusion of extracellular second messengers).

Another important aspect is that inhibition of p38MAPK is effective to abolish PV-IgG-induced epidermal blistering in *ex vivo* human skin models whereas antibodies against Dsg3 such as AK23 or in mucosal-dominant PV-IgG fractions are not sufficient to induce skin blistering, although they activate p38MAPK (152, 182). This suggests that p38MAPK for epidermal blistering is required but not sufficient. In a mucosa model, inhibition of p38MAPK did not block blistering although AK23 in mucosa activated p38MAPK indicating that the relevance of signalling pathways in pemphigus is also tissue-specific (15). All these results indicate that the clinical phenotype of PV with mucosal erosions and deep epidermal blistering on one hand and PF characterised by superficial acantholysis on the other hand are unlikely to be caused by different expression pattern of Dsg3 and Dsg1 alone as suggested by the Dsg compensation hypothesis. Rather, the mechanisms regulating cell adhesion in mucosa and epidermal layers appear to be different and to be regulated by Dsg3 and Dsg1 (3, 150).

Finally, it has to be noted that the observation of Dsg3 and Dsg1 to regulate keratinocyte adhesion and function *via* different mechanisms is also in line with the different phenotypes of mouse models deficient for these molecules. Dsg3-deficient mice besides hair-loss display rather mild erosions in the skin with PV-typical histology, which have been observed to spontaneously heal within several days, but also suffer from eye involvement in conjunctiva and cornea (56, 119, 224). The phenotype may be explained by the observation that in both affected and unaffected skin p38MAPK activity was up-regulated, which was found to be required for wound healing (224). This shows that Dsg3 supports epidermal integrity at least in part *via* regulation of signalling pathways controlling cell adhesion. In contrast, Dsg1-deficient mice lose their superficial epidermis during birth and all die within 24 h due to complete loss of epidermal barrier properties (125). Because TJ morphology was altered in heterozygous mice, it is likely that the function of Dsg1 *via* Erbin to control EGFR-mediated TJ assembly in the superficial epidermis is involved also (230–232) (**Figures 2–5**).

THE CENTRAL ROLE OF p38MAPK TO REGULATE DESMOSOME TURN-OVER

For some signalling pathways the role in pemphigus pathogenesis and the mechanisms underlying loss of desmosomal adhesion became clearer during the last two decades.

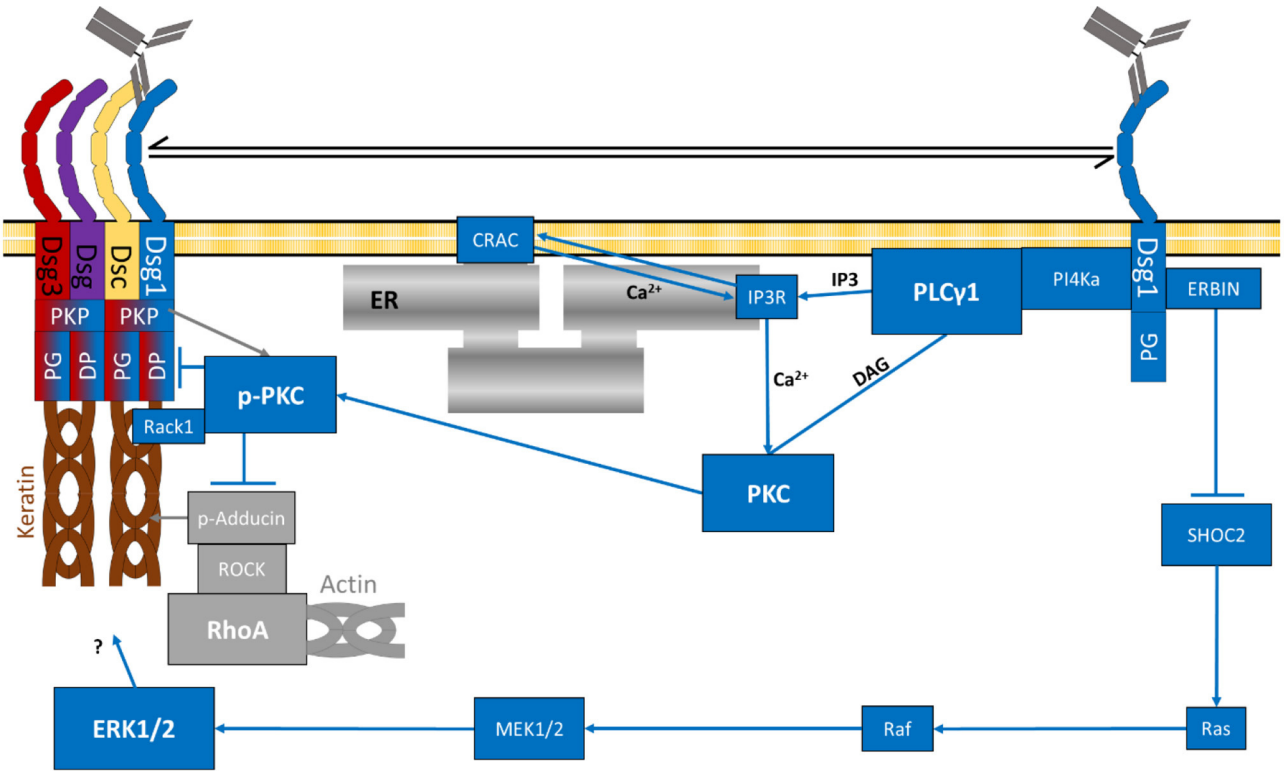

FIGURE 4 | Schematic depiction of specifically Dsg1-dependent signalling mechanisms (grey RhoA complex represents a potential rescue pathway). The specific target of Erk1/2-Elk1 is not known.

An important molecule contributing to pathogenesis of pemphigus is p38MAPK, a protein involved mostly in the reaction to external stressors, including oxidative (233, 234), thermal and chemical stress (234, 235) but can also be activated by growth factors (236). Upon activation by phosphorylation at threonine residue 180 and tyrosine 182 (225, 237–239) p38MAPK activates other kinases further downstream and finally impacts cell growth, differentiation (240), migration as well as wound healing (224) possibly also modulating gene expression (235) and was also reported to directly phosphorylate keratins in keratinocytes and enterocytes (241–243).

p38MAPK is activated by PV-IgG (225, 237) as well by PF -IgG (226) in keratinocytes *in vitro* and in mouse skin *in vivo*. p38MAPK is associated with both intra- as well as extradesmosomal Dsg3 (187) and is phosphorylated in a protein complex with Dsg3 and PG (216). p38MAPK then *via* MAPKAPK2 (56, 244) activates HSP27 (225, 237, 245). In skin biopsies of PV and PF patients, activation of p38MAPK (238), MAKAPK2 (244) as well as HSP27 (238) were confirmed also. HSP27 regulates both actin (236) as well as intermediary filament (246) dynamic restructuring. Moreover, p38MAPK is associated with the keratin cytoskeleton (151, 161, 216, 237).

To delineate the role of p38MAPK in pemphigus pathogenesis it was shown that specific inhibition of p38MAPK or its downstream target MAPKAPK2 are sufficient to reduce loss of adhesion in keratinocytes *in vitro* (221, 247), depletion of both extra- and intradesmosomal Dsg3 (132, 233, 244) from both the soluble and insoluble fractions (132) and the activation of and reorganisation of the cytoskeleton by HSP27 (237) upon PV-IgG treatment. In murine skin *in vivo*, p38MAPK inhibition was sufficient to prevent skin blistering and activation of HSP25, the murine analogue to human HSP27, in the skin (225, 226) upon PV- or PF-IgG treatment. In human skin *ex-vivo*, inhibition of p38MAPK ameliorated the pathogenic effects of PV-IgG, including skin blistering, Dsg3 fragmentation, reduction of desmosome number and size, membrane detachment and keratin retraction (182). In contrast, inhibition of p38MAPK was protective against Dsg1 clustering but did not prevent acantholysis induced by PF-IgG (113).

However, it is currently unclear how exactly p38MAPK is activated after antibody binding to Dsg1 and Dsg3. One study reported activation with two peaks in activity at different points in time, which was different *in vivo* vs. *in vitro* as well as between PV- and PF-IgG. It was proposed that both peaks would most likely cause different cellular responses (248). In other experiments, different peaking patterns were reported (249) or experiments were only performed at specific time points (233) making it impossible to directly compare many of these results (**Figures 2**, **5**).

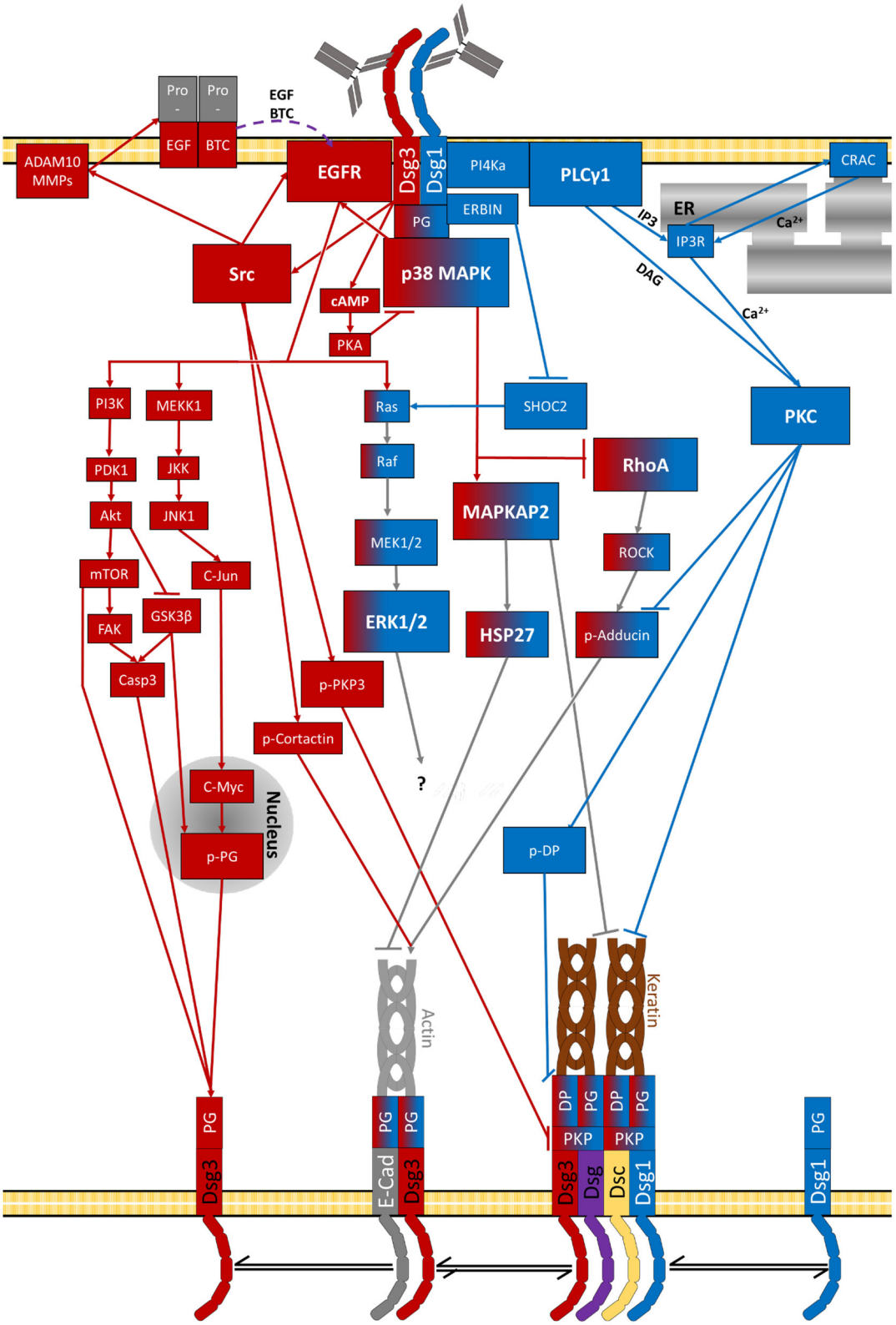

FIGURE 5 | Signalling pathways activated by pemphigus-IgG (purple lines indicate diffusion of extracellular second messengers). The specific target of Erk1/2-Elk1 is not known.

Rho A/ADDUCIN-MEDIATED DESMOSOME ASSEMBLY

One pathway by which p38MAPK could deplete Dsg3 from intra- and extradesmosomal pools is interference with RhoA/adducing-mediated assembly of desmosomes. Reorganisation of the actin cytoskeleton was observed upon treatment with both PV-IgG and PF-IgG (171, 237). RhoA was shown to induce reorganisation of the actin cytoskeleton (157) and is inactivated following PV-IgG and PF-IgG treatment (227). This mechanism appears to be important in pemphigus because pharmacologic activation of RhoA inhibited epidermal blistering in response to PV-IgG and PF-IgG in human skin *ex vivo* and inactivation of Rho-GTPases induced epidermal splitting similar to autoantibodies (227, 250). It was demonstrated that inhibition of p38MAPK abolished RhoA inactivation after PV-IgG treatment identifying RhoA as another downstream target of p38MAPK (127, 227). PKP2 was shown to prevent the localisation of RhoA to cellular interfaces having similar effects as RhoA inactivation (190). RhoA activity was furthermore linked to possible apoptotic signalling in pemphigus (251). The effects of RhoA inactivation include reduced incorporation of DP into the desmosomal plaque (190), reduced anchorage of the desmosomes to the keratin cytoskeleton (227) and depletion of Dsg3 and Dsg1 finally leading to loss of cell adhesion (250, 252–254).

All these effects can be explained by the fact that RhoA *via* Rho kinase regulates the actin binding protein adducin (192). Adducin is essential for cell adhesion in keratinocytes by controlling the incorporation of Dsg3 into desmosomes (193). Other actin binding proteins such as cortactin, which have been shown to be involved in pemphigus pathogenesis, also bind to extra-desmosomal Dsg3 and thus may participate in desmosome assembly (191). Thus, it is possible that inhibition of desmosome assembly *via* p38MAPK-mediated inactivation of Rho family GTPases and actin binding proteins is a general paradigm in pemphigus pathogenesis (**Figures 2**, **5**).

PLAKOGLOBIN ACTS AS SIGNAL TRANSDUCER AND TRANSCRIPTION FACTOR IN PEMPHIGUS

As described above, PG is essential for the assembly of desmosomes (255–257) and cellular adhesion (8, 256, 258) e.g., as part of the Dsg3-PG complex (187). This complex is later incorporated into the desmosomes and becomes insoluble *via* interaction with Dsg1 (259). It was shown, that PV-IgG impairs the distribution of PG (260–262) and its association with Dsg3 (263). As described this is a result of PG endocytosis (218, 260). It was shown that the composition of the desmosomes and cellular adhesion and keratin filament integrity is largely dependent on PG (218, 261). Loss of PG activated p38MAPK as discussed above (218). However, PG was reported to contribute to both desmosomes and adherents junctions (255, 256) but is also found free at the cell membrane as well as in the cytosole (130) and even in the nucleus (218) of keratinocytes. It is thus most likely displaying an even broader range of functions. The localisation of PG is regulated by phosphorylation *via* GSK3β and thus inhibitors of this pathway were shown to be protective against PV-IgG-induced skin blistering *in vivo* (264). By its relocalization to the nucleus, PG also regulates gene expression in pemphigus patients. This role is distinct from its function as a plaque protein (218). PG increases expression of urokinase-type plasminogen-activator receptor (uPAR) at the cell membrane. uPAR was described to be involved in wound healing and cellular reorganisation at the cell membrane (262). Nuclear PG also increased expression of the proto-oncogene c-Myc, which ultimately leads to hyperproliferation, reduced cell adhesion and eventually skin blistering in pemphigus patients (264, 265). This increase of nuclear c-Myc is characteristic for PV and is not observed for other skin diseases including PF, again highlighting that for both variants of pemphigus different signalling pathways are activated. It was observed that c-Myc activity is already increased very early in the pathogenesis and does correlate with disease severity (265). Very recently, inhibitors against the signalling cascade PI3K/PDK1/Akt, which regulate GSK3β, have been shown to be protective against skin blistering *in vivo* and *ex vivo* human skin and also inhibition of the down-stream molecule mTOR was shown to protective in mice (229, 266). Therefore, it is possible that this signalling pathway regulates desmosome turn-over also by mechanisms different from PG (**Figures 3**, **5**).

THE Src/ADAM10/EGFR RECEPTOR PATHWAY IN PEMPHIGUS

Src activated rapidly and in a Dsg3-dependent manner (267) after PV-IgG treatment (249). Src activity was reported to cause loss of Dsg3 from the membrane, phosphorylation of PKP3, thereby destabilising the desmosomal plaque (268) and to induce keratin retraction (249). These changes cause loss of adhesion *in vitro* (191, 268) and skin blistering in mice *in vivo* (66, 172, 191, 266). In peri-lesional skin of pemphigus patients an increased Src activity was observed. In contrast to that, in lesional skin its activity was notably reduced, indicating an activation of Src before akantholysis (267). However, in human skin Src inhibition was not completely effective to prevent acantholysis caused by PV-IgG fractions including higher anti-Dsg1 IgG concentrations. Because Src was no longer activated after 24 h and Src inhibition was only protective during timeframes when Src was active (191), it can be assumed that Src plays a less central role in pemphigus pathogenesis and likely contributes during the initial phase only when the extradesmosomal Dsg3 is depleted. In addition to its pathogenic role, Src is also essential for the Ca^{2+}-dependent incorporation of Dsg3 into desmosomes by stabilising the complex of E-cadherin together with Dsg3, potentially *via* cortactin (187, 191). Thus, Src seems to play an interesting dual role by stimulating desmosome formation under basal conditions but causing desmosome disassembly under pathogenic conditions.

One target molecule of Src in pemphigus pathogenesis appears to be EGFR which was shown to interact with (8, 269) and to be activated *via* Src (228, 249, 270). EGFR inhibition was shown to

be protective against effects induced by PV-IgG and PF-IgG *in vitro* and *in vivo* (228, 271–273). EGFR is an important central molecule of a complex network of signalling pathways (269) and can be activated by ligands like the EGF or TGF but also in a ligand-independent manner *via* phosphorylation by p38MAPK and formation of intracellular signalling complexes (269, 270, 272, 274). EGFR was shown to regulate cellular adhesion and inhibiting it's basal activity promotes localisation of PG and DP to the desmosomal plaque and strengthens cellular adhesion (272, 275). Furthermore, it was demonstrated to be involved in pemphigus signalling (249, 272, 273) but not to be crucial because EGFR-independent loss of keratinocyte adhesion after incubation with PV-IgG has been observed as well (217). EGFR activity leads to endocytosis of Dsg3 and PG causing loss of adhesion (272, 273).

EGFR downstream signalling is broad and the exact mechanisms involved in acantholysis are not perfectly clear but may involve PI3K and ERK which recently were associated with pemphigus pathogenesis (152, 215, 229). PI3K, acting *via* PDK1/Akt/mTOR/GSK3β on nuclear localisation of PG may cause hyper-proliferation and loss of adhesion (269, 276, 277). Similarly, the Ras/Raf/MEK/ERK pathway is involved in the regulation of cell proliferation vs. differentiation and causes loss of adhesion as well (228, 230, 269, 278–281). Another possible target is JNK1 impacting cell proliferation and migration (269, 282, 283).

Besides direct activation of EGFR by phosphorylation at Y845, Src is also involved in the release of activating EGFR ligands in response to pemphigus autoantibodies (66, 228). In this context, metalloproteinases (MMPs) are involved which can cleave cellular proteins and thus convert proteins to active forms, release second messengers or cleave and inactive proteins. They are well-known to be involved in the pathology of bullous pemphigoid (284). It was furthermore reported that in PV and bullous pemphigoid levels of serin proteinases activity were increased in patients' serum and blister exudates whereas MMP inhibitors were reduced (285). MMP9 was reported to be significantly upregulated in PV model *in vivo* and *in vitro* (286). Recently, ADAM10 was reported to be directly involved in pemphigus pathogenesis (66). ADAM10 is activated downstream of Src thereby modulating EGFR activation *via* release of EGF and Betacellulin (BTC). Interestingly, the activation of ADAM10 is dependent on the autoantibody profile and restricted to antibodies targeting Dsg1 and Dsg3. In contrast, additional autoantibodies against Dsc2 and 3 triggered a more severe and earlier acantholysis independent of ADAM10 (66) (**Figures 3**, **5**).

Dsg1 REGULATES EGFR SIGNALLING TOWARDS ERK WHICH MAY BE INVOLVED IN PEMPHIGUS

Dsg1 is a very important antagonist of EGFR signalling. EGFR promotes cell migration and proliferation in the basal layer of the epidermis. In contrast, Dsg1 expression in superficial epidermis shifts the gears more towards keratinocyte differentiation. This antagonistic role is a result of at least one shared pathway. While EGFR is known to activate ERK1/2, Dsg1 is a known suppressor of this signalling pathway (215, 228, 230, 279–281, 287). This suppression is mediated *via* the Dsg1-Erbin-SHOC2-complex which interferes with EGFR-induced activation of MEK1/2 and ERK1/2 (228, 279–281). Therefore, this pathway represents a very important crossing point between Dsg3- and Dsg1-dependent signalling and a protective role of Dsg1 against signalling induced by anti-Dsg3 autoantibodies in pemphigus can explain why in mucosal PV the epidermis is not affected although Dsg3 distribution is severely altered (149, 178, 179). In this line of thoughts, anti-Dsg1 antibodies in PV would shut off the inherent suppression on ERK *via* Erbin-SHOC2 and thereby activate ERK in the absence of EGFR activation as observed following incubation with PV-IgG and PF-IgG (152, 228). This hypothesis is also in line with the inefficiency to prevent loss of adhesion by EGFR suppression in presence of higher concentrations of anti-Dsg1 autoantibodies, which releases the suppression of Erk1/2 signalling (217) (**Figures 3**, **5**).

Dsg1-MEDIATED Ca^{2+}-SIGNALLING IS IMPORTANT FOR EPIDERMAL BLISTERING IN PEMPHIGUS

Relatively early in pemphigus research, PV-IgG treatment was demonstrated to induce activation of PLC and Ca^{2+}-influx *via* IP3R (156, 160). More recently, direct interaction of DSG1 and PI4K (220, 288, 289), an upstream kinase of PLC (290), was reported. For PLC, an interaction with both Dsg1 and Dsg3 was shown (220). Activated PLC generates the second messengers IP3 and DAG (156, 172) which leads to a release of Ca^{2+} from the endoplasmic reticulum (ER) to the cytoplasm (291). Stim1 and Orai1 are two ubiquitously expressed proteins, which together form the so-called CRAC (Ca^{2+}-release-activated-channel). Stim 1 is located at the membrane of the ER (292) where it serves as a sensor for the Ca^{2+} concentration. Upon low Ca^{2+}, Stim1 contacts Orai1, located at the plasma membrane, and both together from a Ca^{2+}-specific channel causing Ca^{2+} influx and replenishing the Ca^{2+} stores of the ER (293–298). In line with this, inhibition of Ca^{2+} signalling *via* inhibition of either PI4K, PLC, IP3R or CRAC was sufficient to ameliorate pathogenic effects of pemphigus autoantibodies *in vitro* and inhibition of PLC and IP3R abrogated skin blistering in human skin *ex vivo* (220).

DAG and Ca^{2+} released by these mechanisms are activators of PKC (195, 299), causing its redistribution to cell contacts (200, 299). PV-IgG was demonstrated to induce PKC activation as well (127, 152, 172, 208, 300). The exact mechanism of action of PKC is yet unknown, however, it was observed that PKC interacts with the keratin filaments (161, 208, 301) and is regulated by PKP1 (207). PKC was furthermore shown to phosphorylate DP which causes destabilisation of the desmosomal plaque (197, 208, 209). Inhibition of PKC reduces the PV-IgG-induced depletion of Dsg3 (127), keratin retraction (208) and loss of cell adhesion (127, 152) *in vitro* and *in vivo* (127, 172) and skin blistering in mice *in vivo* (127, 172). PKC thus clearly plays a role in pemphigus pathogenesis. However, since it was reported that inhibition of

PKC was not sufficient to prevent skin blistering in human skin *ex vivo* (215), PKC is probably not the only downstream target of the PLC/Ca^{2+} pathway. Furthermore, it needs to be noted that there are three types of PKCs with various isoforms, activated by different sets of second messengers. More specific, DAG and cytoplasmic Ca^{2+} activate Ca^{2+}-dependent PKC (cPKC), DAG activates novel PKC (nPKC) and translocation activates atypical PKC (aPKC) isoforms, respectively (299). At least cPKCs and nPKCs are downstream targets of PLC and thus may be activated in response to autoantibodies in pemphigus (**Figures 4**, **5**).

PRE-APOPTOTIC CASPASE SIGNALLING REDUCING Dsg1 AND Dsg3 IN PEMPHIGUS

Several receptor-induced signalling cascades can initiate signalling associated with apoptosis. The caspase protein family plays a central role for this signalling. Caspases cleave certain cellular proteins (154) causing morphologic changes such as condensation of the nucleus, fragmentation of the DNA, cell shrinkage (302) and degradation of the cytoskeleton (303, 304). Amongst the proteins which are cleaved by caspases are the desmosomal cadherins and the plaque proteins causing degradation of desmosomes and cytoskeleton (305). The possibility that these apoptotic pathways can be active in pemphigus was demonstrated in several studies (79, 154, 302, 306–310). Apoptosis can occur in the skin (307, 309, 311, 312) as well as the mucosa (308) of pemphigus patients. However, in acantholytic areas apoptotic cell death is not typically observed (154, 177, 178).

It was shown that PV-IgG increases the activity of pro-apoptotic and decreases that of anti-apoptotic proteins in keratinocytes *in vitro* (309, 311, 313) and mice *in vivo* (266, 306, 307). Caspase3 seems to be the most relevant in this context (306, 307, 314). Caspase signalling may be activated by PV-IgG in several ways. Increased amounts of soluble Fas receptor ligands were found in pemphigus patients' sera and in mice after injection of PV-IgG. These induce activation of caspase8 which causes cleavage of Dsg molecules. Inhibition of Fas receptors and depletion of soluble Fas ligand were also shown to be protective in keratinocytes *in vitro* and mouse skin *in vivo* (309, 315). Activation *via* caspase8 was reported as well (316). In some patients anti-mitochondrial autoantibodies were found and it was shown that they can penetrate into mitochondria. These antibodies were reported to cause JNK activation and *via* caspases may induce degradation of Dsg3 (317, 318). Similarly, Dsg1 was also identified as a caspase3 target (319). The activation *via* c-Jun is also supported by the observation that inhibition of p38MAPK signalling, which causes c-Jun activation *via* EGFR, was sufficient to block caspase3 activation after PV-IgG treatment (248). Furthermore, EGFR was reported to induce caspase signalling and apoptosis (273). Thus, signalling by antibodies against Dsg3 may use this Src/EGFR signalling axis to activate caspases *via* PI3K/PDK1/Akt/GSK3β and nNOS/mTOR/FAK suggestive because inhibitors against this pathway have been shown to be protective against skin blistering (229, 266, 320, 321).

Moreover, caspase signalling could also be induced *via* the Ca^{2+} pathway. In this context, a mechanism was described which links the mitochondrial Ca^{2+} specific channel VDAC1 to IP3R *via* so-called mitochondria-associated membranes (MAM). The resulting Ca^{2+} flux was reported to lead to mitochondrial swelling and induction of apoptosis-related signals (322–326). Caspase3 activity can further be linked to p38MAPK, c-Myc (318), and RhoA activity (251). In line with this, several studies demonstrated that activation of p38MAPK, cell dissociation, and keratin retraction in keratinocytes *in vitro* (318) and skin blistering in mice *in vivo* (79, 266, 306, 307, 318) were significantly ameliorated under pharmacological inhibition of caspase activity.

However, the relevance of apoptosis for the pathology of pemphigus is controversly discussed and often attributed to be a secondary effect caused by acantholysis (111, 154). It was demonstrated that acantholysis takes place independent of apoptosis in keratinocytes *in vitro* (154, 318, 327, 328) as well as in pemphigus patient skin (154, 177, 318, 328). One group reported a slight increase of caspase3 *in vitro* and *in vivo* before the appearance of lesions but without any other sings of apoptosis (318). Taken together, all studies indicate a possible role of caspases for acantholysis independent of apoptotic cell death (306, 318). Since it was already found that caspases also influence several processes including proliferation, differentiation and cell cycle regulation (329), it can be concluded that caspases activate secondary pathways which contribute to skin blistering (154, 302). The fact that both Dsg1 and Dsg3 can be cleaved by caspase3 also would provide a mechanism how apoptotic signalling is directly interfering with desmosome turnover (**Figures 3**, **5**).

RESCUE PATHWAYS IN PEMPHIGUS SUCH AS Dsg2 AND cAMP UPREGULATION

Most interestingly, autoantibodies in pemphigus not only cause pathogenic effects leading to loss of keratinocyte adhesion but also induce rescue pathways. One compensatory mechanism is up-regulation of Dsg2 expression in PV patients' lesions (330). It was demonstrated that forced expression of Dsg2 in the upper skin layers of transgenic mice was protective against PF-IgG-induced skin lesions (331). Moreover, in cultured keratinocytes it was shown that expression of Dsg2 (221) similar to Dsg1 (332) was protective against anti-Dsg3-antibody-induced loss of adhesion. Because Dsg2 can undergo heterophilic interaction with Dsg3, which is less sensitive to direct inhibition of autoantibodies targeting Dsg3, it is possible that Dsg2 up-regulation is supporting to maintain cell adhesion in PV (333).

Another protective pathway is dependent on the second messenger cAMP. Increased levels of cAMP were observed after incubation with PV-IgG and a pharmacological increase in cAMP blocked the effects of PV-IgG *in vitro* and skin blistering in mice *in vivo* (334). It was demonstrated that cAMP was protective against activation of p38MAPK which may involve PKA activation (334). This mechanism may be related to a

phenomenon referred to as positive adhesiotropy by which adrenergic signalling in the heart strengthens cardiomyocyte adhesion *via* PKA-mediated PG phosphorylation (210, 212, 214). Recently, a case of a pemphigus patient was reported who was treated with Apremilast, a phosphodiesterase-4 inhibitor which enhances intracellular cAMP levels. Treatment ameliorated the symptoms and led to a decrease in autoantibody titers (335). Thus, the protective effect was attributed to changes in immune cell signalling rather than modulation of keratinocyte signalling.

Most recently it was reported, that mechanical stimulation also plays a very important role for the resistance against pemphigus-IgG-induced effects. Stretching of cells reportedly induced activation of RohA, strengthening the cortical acting network and its crosslinking, leading to increased cell contractility. This was shown to reduce pemphigus IgG induced effects, indicating, that activation of RhoA in pemphigus might represent an insufficient rescue pathway (336) (**Figures 2**, **5**).

CONCLUSION: HOW TO ELUCIDATE A FEASIBLE APPROACH TO STABILISE DESMOSOMAL ADHESION FOR PEMPHIGUS THERAPY?

From this review it can be concluded that some signalling pathways such as p38MAPK, ERK and PLC/Ca^{2+} appear to be more relevant for skin blistering in pemphigus than others. This is supported by a recent study using an unbiased approach by testing a library of 141 small molecule inhibitors in different experimental models of pemphigus which found that inhibition of ERK and p38MAPK signalling was effective to ameliorate PV-IgG-induced loss of cell adhesion (229).

On the other hand, inhibition of p38MAPK in clinical studies was not beneficial for pemphigus patients (271). This maybe explained in part by the fact that inhibition of a central signalling pathway involved in many different functions (234) is associated with severe side effects. It can be envisaged that the situation is not different for approaches modulating ERK or PLC/Ca^{2+} signalling indicating that at least systemic therapy using inhibitors of these pathways are most likely not feasible. Nevertheless, since topical application of Dsg-crosslinking peptides or drugs to increase cAMP were effective in pemphigus mouse models *in vivo* (216, 334) it may also be possible to reduce side-effects by site-specific application of modulators of signalling pathways in the future. Therefore, it is very encouraging that topical application of selumetinib to inhibit MEK1, which is the kinase up-stream of ERK1/2, was protective in the human skin organ culture model (229).

Moreover, identification of signalling mechanisms critical for pemphigus pathology allows to focus on the molecular targets of the associated signalling molecules. Because PKC downstream of PLC/Ca^{2+} has been shown to regulate desmosome stability *via* phosphorylation of DP, the modulation of DP cytoskeletal anchorage may be a promising target for therapy. Similarly, given that p38MAPK induces pathogenic effects *via* inhibition of RhoA, which *via* Rho kinase and adducin phosphorylation is important for desmosome assembly (190, 192, 193, 227), therapeutic approaches to stimulate desmosome assembly should be characterised in more detail. Finally, since the small inhibitor library helped to identify a role in keratinocyte adhesion regulation for VEGFR2, TrkA, PI3K, and PDK1 (**Table 2**), new candidates for treatment options have been elucidated as well (229). Therefore, we believe that studies on agents for additional treatment of patients suffering from the acute phase of pemphigus or facing relapses are of high importance in the future.

AUTHOR CONTRIBUTIONS

TS: concept, original draft, figure design, and revision. JW: concept and revision. Both authors contributed to the article and approved the submitted version.

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MINI REVIEW
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Bruton Tyrosine Kinase Inhibition and Its Role as an Emerging Treatment in Pemphigus

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Bruton Tyrosine Kinase (BTK) has a key role in multiple pathways involved in inflammation and autoimmunity. Therefore, BTK has become a new therapeutic target for a group of hematologic and autoimmune disorders. The pharmaceutical industry has invested in the clinical development of BTK inhibitors during the last decade. Ibrutinib, for example, which was the first BTK inhibitor to be used in clinical trials, has two approved indications, mantle cell lymphoma and chronic lymphocytic leukemia, and remains under evaluation for additional indications. Rillzabrutinib (PRN1008) is a new, highly potent and selective inhibitor of BTK. Early studies performed in canine pemphigus demonstrated effectiveness. A proof-of-concept, multicenter, phase 2 trial has recently showed the efficacy and safety of oral rilzabrutinib in pemphigus vulgaris. In this mini review, we present evidence regarding the mechanisms affected by BTK inhibition and the concept of BTK inhibition as an emerging new treatment in pemphigus.

Keywords: Bruton's tyrosine kinase, rilzabrutinib, BTK inhibition, Pemphigus vulgaris, pemphigus foliaceus

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Bruton Tyrosine Kinase (BTK) has a key role in multiple pathways involved in inflammation and autoimmunity. Therefore, BTK has become a new therapeutic target for a group of hematologic and autoimmune disorders. The pharmaceutical industry has invested in the clinical development of BTK inhibitors during the last decade. Ibrutinib, for example, which was the first BTK inhibitor to be used in clinical trials, has two approved indications, mantle cell lymphoma and chronic lymphocytic leukemia, and remains under evaluation for additional indications (1).

In this mini review, we present evidence regarding the mechanisms affected by BTK inhibition and the concept of BTK inhibition as an emerging new treatment in pemphigus.

WHAT IS BTK

Bruton tyrosine kinase (BTK) is an enzyme with a major role in both the innate and the adaptive immune responses. It is part of the signaling pathway of most white blood cells, apart from T cells and plasma cells. BTK contributes significantly in the proliferation and differentiation of B-cells. It also has a role in myeloid cell inflammatory cytokine production. Thus, BTK is being investigated nowadays as a promising target for the treatment of immunological disorders and B-cell hematological malignancies.

DR. BRUTON

In 1952, the American pediatrician Dr. Ogden Bruton first described a case of an 8 year-old boy who presented with recurrent bacterial sepsis, osteomyelitis and otitis (2). The patient's failure

to produce antibodies was attributed to the lack of a gamma globulin fraction. This was the first case of X- linked agammaglobulinemia (XLA), a disease manifested by markedly decreased serum immunoglobulins and almost total absence of B cells in the peripheral blood. The defect in XLA was initially mapped in 1986 (3). In 1993, it was found that XLA was due to mutations in the gene encoding a cytoplasmic tyrosine kinase which was named Bruton tyrosine kinase (4). It is estimated that 1 in 150,000 males is born with this BTK gene deficiency.

A less severe X-linked immunodeficiency has been described in mice. An important observation was that these mice were resistant to acquiring lupus (5) and collagen induced arthritis (6).

AGAMMAGLOBULINAEMIA

X-linked agammaglobulinemia (XLA) is an immunodeficiency typically presented with a failure to produce mature B lymphocytes and accompanied with a failure of Ig heavy chain rearrangement. The defect in XLA resides in Bruton tyrosine kinase (BTK), also known as B cell progenitor kinase (BPK) or agammaglobulinemia tyrosine kinase (ATK), a key regulator in B-cell development (7). There is a genetic heterogeneity in XLA. Most cases are caused by mutation in the SH3 Domain Containing Kinase Binding Protein 1 (SH3KBP1) gene on chromosome Xq22.

FAMILY OF TYROSINE KINASES

Tyrosine kinases are a large multigene protein family related to cancer and many diseases. More than 90 kinase genes have been identified in the human genome.

The tyrosine kinases are divided into two main classes, receptor and non-receptor tyrosine kinases.

The transmembrane receptor-linked kinases are 58 and distributed into 20 subfamilies. The non-receptor tyrosine kinases are 32 and placed in 10 subfamilies. This family of kinases function in multicellular pathways. Signals concerning cell differentiation, growth, adhesion, and death are transmitted through them and there is evidence that they play a role in cancer and in many diseases (8).

B—CELL MALIGNANCIES AND BTK INHIBITION

BTK acts as an essential kinase of B-cell–receptor signaling, intercedes actions within the tumor microenvironment and enhances the proliferation and survival of malignant cells. BTK is a driving factor for the activation of various active pathways of malignant cell viability, including the AKT, extracellular signal–regulated kinase (ERK), and nuclear factor kappa light-chain enhancer of activated B cells (NF-κB) pathways (1, 7).

Ibrutinib, an oral covalent BTK inhibitor, has substantiated clinical efficacy in newly diagnosed as well as relapsed lymphoma patients compared to traditional chemotherapy (9). The first paradigm of therapeutic benefit targeting BTK was the rapid approval of ibrutinib by the US Food and Drug Administration (FDA) as a therapeutic option for chronic lymphocytic lymphoma/small lymphocytic lymphoma (CLL/SLL) and MCL (mantle cell lymphoma) in 2014 and 2013, respectively. It was subsequently approved as monotherapy in patients with lymphoplasmacytic lymphoma (LPL)/ WM (Waldenstrom's macroglobulinemia) and marginal zone lymphoma (MZL) in 2015 and 2017, respectively (10). Its safety profile has been a concern due to extended off-target kinase inhibition including Tec kinases, epidermal growth factor receptor (EGFR) and interleukin 2-inducible T cell kinase (ITK) (11). Associated side effects, such as atrial fibrillation, bleeding, infections, and arthralgias have led to treatment discontinuation in many patients with CLL.

The next generation of BTK inhibitors aimed to show less off-target inhibition and improved tolerability with comparable efficacy. Acalabrutinib, a selective covalent BTK inhibitor, was approved as a treatment option for CLL by the FDA in 2019 (12). Other next generation BTK inhibitors, such as zanubrutinib, tirabrutinib, vecabrutinib, and fenebrutinib, are currently studied (13).

PEMPHIGUS AND SIGNALING PATHWAYS

In pemphigus, in genetically susceptible individuals, an autoimmune reaction is driven by CD4 autoreactive T lymphocytes which are specific for Dsg molecules and induce the production of anti Dsg- antibodies by B lymphocytes (14).

In pemphigus vulgaris (PV) and pemphigus foliaceus (PF), target antigen specific autoantibodies induce keratinocyte detachment (acantholysis) and blister development.

Dsg1 is mainly expressed in the upper layers of the epidermis while Dsg3 is mostly expressed in the lower. The Dsg1/Dsg3 compensation theory explains why skin and mucosa are not equally affected by the target antigen specific autoantibodies in different pemphigus forms (15). In PV, for example, sera with Dsg3- specific IgG do not induce suprabasal acantholysis because Dsg1 is sufficiently expressed and compensates epidermal adhesion in the absence of Dsg3. Anti-Dsg3 IgG causes loss of adhesion in the mucous membranes as there is a low expression of Dsg1, which is insufficient to compensate disabled Dsg3 adhesion (15).

The exact cascade resulting in pemphigus acantholysis is still under research. Three major events following the binding of anti-Dsg IgG have been described. The direct interference with Dsg transinteraction through homophilic and heterophilic binding of Dsg molecules to Dsg and Dsc molecules on neighboring cells is a phenomenon termed steric hindrance. Dsg transinteraction via steric hindrance and altered outside-in-signaling by pemphigus autoantibodies leads to loss of desmosomal integrity and blister formation (14, 15).

Pemphigus is also known as a desmosome-remodeling disease because remodeling of Dsg expression on the cell surface results in internalization and depletion of Dsg. This depletion process of Dsg leads to acantholysis mainly induced by a direct interference of trans-interaction of Dsg. Desmosomes become fragile as they

lose their adhesive properties at protein levels and are more susceptible for subsequent depletion processes (16).

Activation of keratinocyte intracellular signaling pathways is a crucial component of pemphigus IgG-mediated acantholysis. The signaling events impair the cytoskeletal architecture. These mechanisms don't apply equally for Dsg1 IgG- and Dsg3 IgG-binding. Upon targeting of Dsg1, Ca2+ influx is induced and therefore the extracellular-signal-regulated kinase (ERK) pathway is activated. On the other hand, after binding of Dsg3-specific IgG, signaling via p38MAPK occurs within the epidermis but not in mucosal tissues, and SRC family (non-receptor tyrosine kinases) and EGFR pathways are activated (17). According to the current literature, it is debated whether non-Dsg antibodies contribute to pemphigus phenotype (18–20).

In studies where PV Dsg-3 autoantibodies were used to initiate desmosome signaling in human keratinocyte cell cultures, it has been observed that PV IgG binding to Dsg3 activates desmosomal signal transduction cascades resulting in p38 mitogen-activated protein kinase (MAPK) and Heat Shock Protein—HSP27 phosphorylation and cytoskeletal reorganization. Both p38MAPK and HSP27 regulate components of the cytoskeleton including actin and intermediate filaments. These observations supported a mechanistic role for signaling in PV IgG-induced acantholysis. Consequently, it was suggested that targeting desmosome signaling via inhibition of p38MAPK and HSP27 phosphorylation may act as a novel targeted treatment for PV and other desmosome-related blistering diseases (21).

It was found also that PV IgG causes internalization of cell-surface Dsg3 into endosomes as early as 4 h. This cell-surface Dsg3 internalization and depletion was blocked by p38MAPK inhibition in pemphigus mouse models (22). The same authors demonstrated two peaks of p38MAPK activation in pemphigus keratinocyte tissue culture and mouse models. Examination of the temporal relationship of p38MAPK phosphorylation and apoptosis showed that apoptosis occurs at or after the second peak of p38MAPK activation. The ability of inhibitors of p38MAPK to block activation of the proapoptotic proteinase caspase-3 and the time course of p38MAPK activation and apoptotic markers suggest that activation of apoptosis is downstream to p38MAPK activation in pemphigus acantholysis. Therefore, blocking caspase-dependent Dsg degradation may augment cell-cell adherence and reduce the acantholytic effects of pathogenic IgG (23).

The activation of intracellular signaling events mediated by p38MAPK and HSP27 was also observed in pemphigus patient skin and was analogous to the increased phosphorylation of those two proteins (24). All the above autoantibody-triggered cellular signaling pathways are considered as pathogenic co-mechanisms in pemphigus but, none can solely induce acantholysis.

In pemphigus lesions dermal infiltrates contain interstitial and perivascular neutrophils and eosinophils evolving from innate immune response activation. Hence, not only adaptive but also innate immunological pathways may provide therapeutic targets.

The concept behind the employment of BTK inhibition in pemphigus is that a selective BTK inhibitor has the potential to focus on multiple pathways involved in autoimmunity, like modulation of BCR-mediated B-cell pathways, inhibition of FcεR-induced cytokine release from monocytes and macrophages, mediator release, neutrophil migration and FcγR-induced mast cell degranulation.

IBRUTINIB REPORT FOR A PARANEOPLASTIC (PNP) CASE

The effectiveness of BTK inhibition in pemphigus was first reported in a 51-year patient who suffered from chronic lymphocytic leukemia and developed paraneoplastic pemphigus (PNP). CLL disease control was achieved with ibrutinib and together there was a significant improvement of his pemphigus lesions. This was the first indication that ibrutinib may be added as a treatment option for pemphigus (25).

RILZABRUTINIB BIOLOGY

Rillzabrutinib (PRN1008) is a highly potent inhibitor of BTK with unique reversible covalent binding that has the potential to improve the safety profile compared with irreversible BTK inhibitors such as ibrutinib.

Rilzabrutinib binds in a covalent manner, enhancing selectivity by forming a chemical bond to a particular cysteine residue present in BTK. The addition of the covalent binding site increases the selectivity of rilzabrutinib for BTK vs. other kinases and reduces the dissociation rate to provide BTK binding dynamics that allow for convenient dosing. Reversible BTK binding allows rapid restoration of BTK function following rilzabrutinib withdrawal and reduces off-target effects (26).

A significant advantage of rilzabrutinib is that only short exposure is required to get a clinical benefit (27). Low systemic exposure reduces side effects, increases tolerability and promotes effectiveness and drug survival.

Rilzabrutinib acts on a range of immune cells. It inhibits B cell activation through inhibition of the BCR. However, it doesn't cause B cell depletion or cellular cytotoxicity.

This is a key difference from current therapies like rituximab because it doesn't provoke prolonged immune suppression. Additionally, rilzabrutinib rapidly inhibits Ab-mediated immune cell activation through Fc-receptor signaling. The activation of macrophages and monocytes via cross-linking of IgG and FcgR is a BTK dependent function; the ability of rilzabrutinib to prevent IgG mediated FcgR activation was illustrated in rodent models of lupus nephritis and immune thrombocytopenia (ITP). Rilzabrutinib has shown a unique dual Ab mechanistic approach over the standard treatments for immune-mediated diseases, based on its fast action on halting self-reactive Ab signaling and its longer-term impact on the formation of new autoantibodies (27).

Inhibition of BTK by rilzabrutinib also disrupts the signaling pathway for neutrophil recruitment. This alleviation of neutrophil recruitment may also be beneficial for patients with immune-mediated disorders, because of the reduced

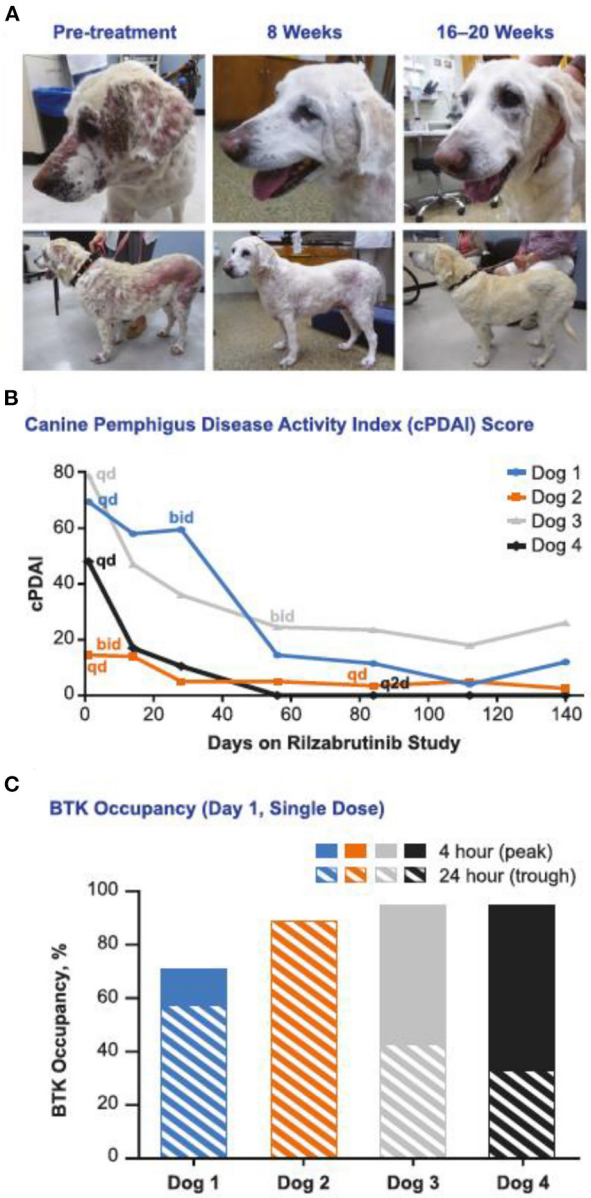

FIGURE 1 | Rilzabrutinib monotherapy (without CSs) was effective for treatment of canines with naturally occurring PF. **(A)** a 13-y-old, yellow Labrador retriever with an initial cPDAI score of 69.5 began with a 500 mg/d dosage of rilzabrutinib and was escalated to 500 mg every 12 h at week 4. Significant decreases in lesions and regrowth of nearly all her coat was achieved by week 20. **(B)** cPDAI scores over time showed 77–100% improvement with rilzabrutinib dosing as monotherapy ($n = 4$ dogs). **(C)** BTK occupancy measurements ranged from 50 to 93% at the 4-h (peak) and 24-h (trough) timepoints [adopted by Langrish et al. (27)].

neutrophil—produced reactive oxygen species and associated tissue damage (28).

Rilzabrutinib does not affect the normal platelet aggregation and therefore, there is not an increased risk of bleeding as with irreversible BTKis, e.g., ibrutinib. Moreover, it isn't active on B cells which are stimulated via a non-BTK–mediated pathway or on T cells, keeping other receptors and signaling pathways that are vital for maintaining immunity, antiviral responses and lymphocyte counts in immune-compromised patients (27).

It is important that rilzabrutinib specifically suppresses inflammatory cellular activities in cells activated in immune-mediated diseases like mast cells, basophils and neutrophils without killing them. This action is fast, as evidenced by rilzabrutinib's rapid efficacy in a mouse model of immune thrombocytopenia (ITP) and in rats with collagen-induced arthritis (CIA) (27).

In a phase 1 study in 62 healthy volunteers, rilzabrutinib was well-tolerated following oral administration. No severe adverse events occurred during the trial. The most common adverse events were mild and mainly were associated with the gastrointestinal system (29). Another BTK inhibitor-PRN473 showed a promising response in canine pemphigus foliaceus (PF) but had lower bioavailability in human than PRN1008 (30). In a Phase II canine pemphigus foliaceus study with PRN1008, all dogs showed reduction in lesions and canine PDAI score during the first 2 weeks of treatment and continued to achieve near complete remission by 20 weeks (31). Rilazabrutinib was granted Orphan Drug Designation by FDA for the treatment of patients with PV, after the encouraging results of a phase II open-label cohort study examining rilzabrutinib in adult patients with PV (**Figure 1**) (32).

The results of this proof-of-concept, multicenter, phase 2 trial examining the efficacy and safety of oral rilzabrutinib in PV were published recently (33). Rilzabrutinib alone, or in combination with low CS doses was safe with fast clinical response in PV patients (33). Following this proof-of-concept trial, a phase 3 pivotal study (PEGASUS; NCT03762265) of rilzabrutinib vs. placebo with CS taper is underway for PV.

CONCLUSION

BTK inhibitors offer a new paradigm for the treatment of autoimmune diseases and AIBD in particular, with pemphigus having the most compelling unmet need, since they potentially act much faster via the innate immune system, compared with rituximab. Early studies performed in canine pemphigus demonstrated effectiveness. A proof-of-concept, multicenter, phase 2 trial recently showed the efficacy and safety of oral rilzabrutinib in pemphigus vulgaris and a large phase III RCT of rilzabrutinib vs. placebo is currently underway.

AUTHOR CONTRIBUTIONS

All authors contributed in conception, literature search, and writing.

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ORIGINAL RESEARCH
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Topical Application of the PI3Kβ-Selective Small Molecule Inhibitor TGX-221 Is an Effective Treatment Option for Experimental Epidermolysis Bullosa Acquisita

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Class I phosphoinositide 3-kinases (PI3K) have been implemented in pathogenesis of experimental epidermolysis bullosa acquisita (EBA), an autoimmune skin disease caused by type VII collagen (COL7) autoantibodies. Mechanistically, inhibition of specific PI3K isoforms, namely PI3Kβ or PI3Kδ, impaired immune complex (IC)-induced neutrophil activation, a key prerequisite for EBA pathogenesis. Data unrelated to EBA showed that neutrophil activation is also modulated by PI3Kα and γ, but their impact on the EBA has, so far, remained elusive. To address this and to identify potential therapeutic targets, we evaluated the impact of a panel of PI3K isoform-selective inhibitors (PI3Ki) on neutrophil function *in vitro*, and in pre-clinical EBA mouse models. We document that distinctive, and EBA pathogenesis-related activation-induced neutrophil *in vitro* functions depend on distinctive PI3K isoforms. When mice were treated with the different PI3Ki, selective blockade of PI3Kα (alpelisib), PI3Kγ (AS-604850), or PI3Kβ (TGX-221) impaired clinical disease manifestation. When applied topically, only TGX-221 impaired induction of experimental EBA. Ultimately, multiplex kinase activity profiling in the presence of disease-modifying PI3Ki identified unique signatures of different PI3K isoform-selective inhibitors on the kinome of IC-activated human neutrophils. Collectively, we here identify topical PI3Kβ inhibition as a potential therapeutic target for the treatment of EBA.

Keywords: neutrophils, bullous skin diseases, signaling, PI3K, immune-complex induced autoimmunity

INTRODUCTION

Class I phosphoinositide 3-kinases (PI3Ks) are heterodimers consisting of one regulatory and one homologous p110 catalytic subunit. The p110α and p110β isoforms are ubiquitously expressed and mice lacking p110α or p110β die during embryonic development (1, 2). The p110δ subunit is primarily found in leukocytes (3, 4), and has essential roles in development and function of

lymphocytes, mast cells and possibly neutrophils (5–7). The p110γ subunit is highly expressed in the immune system but also in other tissues, including heart and central nervous system (8, 9). Regarding its role in immune homeostasis, aberrant PI3K signaling may lead to hematologic malignancies or chronic inflammatory diseases (10, 11).

In pemphigoid diseases, a group of autoimmune skin diseases, neutrophils are recruited to the sites of autoantibody deposits and are activated by binding to the tissue-bound immune complexes (ICs) (12). Binding to ICs is facilitated by FcγR expressed by neutrophils. Signaling downstream of activating FcγR is initiated by receptor clustering, leading to activation of Src family kinases and subsequently SYK. Next, downstream kinases, including PI3K, are activated (13–15). Neutrophils express all 4 class I PI3K isoforms (16, 17). Of these, PI3Kβ/γ/δ, but not p110α, have been implicated to regulate neutrophil activation. More specifically, PI3Kβ (16) and PI3Kδ (18) were shown to be crucial for IC-induced neutrophil activation, specifically the release of reactive oxygen species (ROS). PI3Kγ controls ROS release from fMLP-stimulated human and murine neutrophils (19), as well as neutrophil migration toward GM-CSF (17). PI3Kδ inhibition also leads to selective impairment of neutrophil functions, including IC-, C5a-, and fMLP-induced ROS release, while no impact on PMA-induced ROS release is observed. Furthermore, IL-8- or fMLP-induced migration are impaired by PI3Kδ inhibition, while C5a-induced migration remains unaffected (18). Furthermore, TNF- and LPS-induced cytokine release from neutrophils (19, 20) and extravasation (7) are controlled by PI3Kδ.

Epidermolysis bullosa acquisita (EBA) is a chronic autoimmune pemphigoid-like disease characterized and caused by autoantibodies targeting type VII collagen (COL7) (21, 22). Two distinct clinical EBA manifestations have been described: (i) mechano-bullous EBA and (ii) inflammatory EBA. Overall, EBA is notoriously difficult to treat—combined immunosuppressive treatment induces remissions after 9 months (23–25). Therefore, the implementation of novel therapeutic strategies would be of great benefit. Insights from model systems of the disease that reflect the inflammatory variant of EBA (26) showed that IC activation of neutrophils is a key prerequisite to induce tissue pathology in EBA (21). In line with this notion, inhibition of class I PI3K has been shown to dampen clinical disease manifestation in pre-clinical EBA models. More specifically, PI3Kβ deficient mice are almost completely protected from induction of experimental EBA by transfer of COL7 antibodies (16), and pharmacological PI3Kδ inhibition has therapeutic effects in immunization-induced EBA (18). The impact of the PI3Kα and γ-isoforms on EBA has, however remained elusive. Systemic blockade of the PI3K signaling pathway is associated with severe adverse events, i.e., neutropenia and pulmonary infections (27). Thus, for the treatment of EBA, topical application of PI3K inhibitors (PI3Ki) would be ideal in terms of efficacy and safety. To address these questions, we here used a panel of 7 PI3Ki with selectivity profiles covering all PI3K isoforms in experimental models of EBA.

Based on the current understanding of autoantibody-induced tissue pathology in the inflammatory variant of EBA (12, 21), where neutrophils migrate form the blood into the skin, form an immunological synapse by binding to the immune complexes located at the dermal-epidermal junction, and ultimately release ROS, we evaluated the impact of the PI3Ki on (i) IL-8-induced neutrophil migration, (ii) IC-induced neutrophil spreading, and (iii) IC-induced ROS release form neutrophils. For pre-clinical translation, the impact of systemic and topical PI3Ki treatment was evaluated in antibody transfer-induced EBA.

MATERIALS AND METHODS

Studies With Human Biomaterial

For isolation of PMNs and peripheral blood mononuclear cells (PBMCs), normal human blood was obtained. Healthy controls gave their written informed consent prior to study participation. All of the experiments using human samples were approved by the local ethics committee (University of Lübeck, Lübeck, Germany, AZ 09-140 and AZ 20-341) and were performed in accordance with the Declaration of Helsinki.

Mice

C57BL/6J mice (Charles River, Sulzfeld, Germany) were bred in a specific pathogen free environment and provided standard mouse chow and acidified drinking water *ad libitum*. Gender-matched mice were used for experimental EBA models at the age of 8–10 weeks. Animal experiments were approved by local authorities of the Animal Care and Use Committee (Kiel, Germany) and performed by certified personnel [AZ122.5(108/08-15)] following the ARIVE guidelines.

Chemicals

If not otherwise stated, all other chemicals were applied from Merck or Sigma. All PI3K blocker were supplied by Selleckchem (Houston, Tx, USA). A list of used drugs including IC_{50} values and concentration of oral administration is prepared as **Supplementary Table 1**. For *in vitro* assays, 10 mM were dissolved in DMSO and further diluted in RPMI media. Final concentration for all *in vitro* assays were 1,000, 100, 10, 1, and 0.1 nM. For all *in vitro* assays, the final DMSO concentration was adjusted to 0.01%. All *in vitro* data were recorded in technical duplicates, and the mean of the results was normalized to the value obtained from stimulated cells without PI3K inhibition.

For *in vivo* experiments (oral administration), PI3K inhibitors were dissolved in 10% DMSO/0.8% methylcellulose/0.1% Tween-80. For topical administration to the ear 100 µl 0.9 mg/ml PI3K inhibitors in DMSO: Acetone, 1: 25 were administered daily by dropping.

Human Polymorphonuclear (PMN) Purification

For the PamGene analysis, FACS, NET formation, cytokine release, and ROS release assays, human PMNs were isolated from whole blood samples using a PolymorphPrep™ (Progen, Heidelberg, Germany) gradient according to the manufacturer's instructions. The purity of the PMNs was evaluated by flow cytometry (MACSQuant® Analyzer 10, Miltenyi) using fluorescent staining with anti-human CD14 (clone HCD14,

Biolegend) and anti-human CD15 antibodies (clone VIMC6, Miltenyi). For all experiments, the purity of PMNs was >85%.

For chemotaxis assay, PMNs were isolated from normal human blood using Ficoll™ (28). In detail, blood was diluted 1:3 in HES/PBS (1:1) and allowed to sediment for 45 min. Supernatant (20 ml) was slowly added to 8 ml of Ficoll™ and centrifuged at 850 × g for 25 min (RT) without braking. The sediment (containing granulocytes) was erythrocyte lysed with Aq. dest. for 45 s, and the reaction was stopped with the same volume of 2xPBS After washing, the cells were diluted to 4×10^6/ml in RPMI 1640 (without phenol red) containing 0.5% bovine serum albumin (BSA).

For spreading assay, human blood is mixed 1: 2 with 1% Polyvinylalcohol at RT, and left at RT for 30–45 min and slowly layered onto Pancoll (PAN biotech, Aidenbach, Germany). The tube is centrifuged for 20 min without brake at 850 × g at RT. The supernatant is discarded and the PMNs are transferred into a fresh 50 ml tube by prior resuspension and erythrocyte lysis with 1 ml of distilled water for a total of 45 s. The lysis is stopped with 2xPBS and the tube is filled up with 1xPBS (without Ca/Mg). After centrifugation, the cell pellet is washed and then resuspended with RPMI 1640 (without phenol red) containing mit Glutamin, 2 g/L NaHCO3,1% FCS, 2 g/L Glucose 25 mM HEPES at a concentration of 2×10^6 cells/ml.

IC-Induced ROS Release From PMNs

A LumiTrack™ high binding 96-well-plate (Thermo Fisher Scientific, Waltham, MA, USA) was coated with immunocomplexes (ICs) consisting of human COL7E-F antigen at a final concentration of 2.5 μg/ml and anti-human COL7 IgG1 antibody at a final concentration of 1.8 μg/ml, as described previously (29). A total of 2×10^5 cells were added per well in the presence/absence of PI3K inhibitors at the indicated concentrations. As controls, antigens or antibodies alone were added to the wells. Just before the measurement was taken, luminol was added to the wells, and the chemiluminescence resulting from the ROS production was measured immediately in a luminescence reader (Perkin Elmer GloMax). The ROS release was measured for 1 s per well, sixty-six times for a period of ∼2 h at a constant temperature of 37°C (29).

Neutrophil Adhesion and Spreading on IC

A 96-well E-plate (ACEA bioscience, Bremen, Germany) was coated with 0.5 μg/well mCOL7 and incubated overnight at 4°C. The E-plate is washed with PBS/1%BSA/0.05% Tween20 and dried for 2 h at 37°C with 5% CO_2. Thereafter, the plate was incubated with 1.8 μg/ml anti mCOL7-IgG for 1 h at 37°C to perform immobilized ICs. The plate is then washed again and incubated with the cell suspension (2×10^6/ml) w/o PI3K inhibitors. IC-stimulated with vehicle cells serve as positive control and the negative control consists of unstimulated cells, antigen or antibodies and cells. Adhesion was measured for 2 h. Neutrophil adhesion was monitored by phase contrast microscopy quantified by real-time impedance measurement by using the xCELLigence system (Roche, Penzberg, Germany) (30). The electrical impedance reflects the surface area covered by cells, and is therefore associated with the immune complex mediated neutrophil adhesion. It is expressed as cell index in arbitrary units. As microscopic control, cells were stimulated in addition with ICs consisting of human COL7E-F antigen at a final concentration of 2.5 μg/ml and anti-human COL7 IgG1 antibody at a final concentration of 1.8 μg/ml for 2 h at 37°C and treated w/o 10 μM of the respective PI3K inhibitors.

IC-Induced Stimulation of PMNs and Determination of Cytokine Concentrations and Toxicity

A high binding 96-well-plate (Thermo Fisher Scientific) was coated with ICs consisting of human COL7E-F antigen at a final concentration of 2.5 μg/ml and anti-human COL7 IgG1 antibody at a final concentration of 1.8 μg/ml, as described previously (29). A total of 2×10^5 cells were added per well w/o PI3K inhibitors at the indicated concentrations. As controls, antigens or antibodies alone were added to the wells. Cells were incubated for 2 h at 37°C in an atmosphere containing 5% CO_2. The supernatant was analyzed by Legendplex (Biolegend, San Diego, USA) for TNF and IL-6 among others (for a list see **Supplementary Table 2**) following the manufacturer's protocol. For FACS analysis, single cells were stained for the following surface markers using standard FACS procedures: anti-CD15 (clone VIMC6) and anti-CD45 (clone H130) both from Biolegend or Miltenyi. For live/dead staining, Annexin V and PI were used in accordance with the manufacturer's protocol. Cells were first gated for scatter (SSC-A/FSC-A) and singlets (FSC-H/FSC-A). The CD45+/CD15+ gates were further analyzed for double-positive staining of PI and Annexin V for the measurement of toxicity/survival ($CD15^{pos}CD45^{pos}PI^{neg}$Annexin V^{neg} cells are identified as live cells). Measurements were performed on a Miltenyi MacsQuant10, and data were analyzed with the MACSQuantify™ Software (Version 2.11).

IL-8-Induced Neutrophil Chemotaxis

Chemotaxis was measured using a 48-well Boyden chamber (NeuroProbe Inc., Cabin John, MD) as described previously (31). The chamber was pretreated as described in the manufacturer's protocol. The bottom wells were blocked for 1 h with blocking buffer (1% BSA in 0.1 M $NaHCO_3$/0.1 M Na_2CO_3, pH = 9) at 37°C. The buffer was carefully removed, and the chamber was dried at 37°C. The bottom wells were filled with 30 μl of 6–12 nmol/L human IL-8 (Peprotech, Hamburg, Germany) in 0.5% BSA/PBS (with Ca/Mg). The chambers were covered with a polycarbonate membrane (pore size, 3 μm; Costar Nucleopore GmbH, Tübingen, Germany) and incubated for 30 min at 37°C. Finally, the top wells received 50–80 μl of the isolated PMNs containing the appropriate concentrations of PI3K inhibitors and were incubated for 1 h at 37°C in an atmosphere containing 5% CO_2.

The assay was stopped by replacing the cell suspension in the upper well for another 5 min to completely detach migrated cells from the bottom side of the filters. Then, filters were removed, and the migrated cells were transferred from the bottom wells to a microtiter plate. Residual cells in the bottom wells were lysed by adding 25 μl of 0.2% hexadecyltrimethylammonium

bromide/0.1% BSA in PBS, and were combined with the cells transferred to the microtiter plate; cell lysis was continued for 15 min. Then, 50 µl of TMB solution (Thermo Fisher Scientific) was added until a color reaction was visible, and the enzymatic reaction was stopped by adding 50 µl 1 M H_2SO_4. The color reaction was determined at 450 nm in a microplate reader. The number of migrated cells was calculated from a standard of lysed cells run in parallel.

Measurement of C5a and IL-8 Release From Keratinocytes

Affinity purified antibodies against hCOL7 E-F (EBA) were isolated from patient immunapheresis material as described (32, 33). Briefly, antigen stock solutions were prediluted to a concentration of 1.5 mg/ml in stock solvent (PBS), then further diluted 1:3 in 0.1 M Borat buffer (pH 9.5) (final volume 12 ml). Antigens were subsequently coupled to NHS-Sepharose Fast Flow[4] columns (GE Healthcare, Chicago, USA) following manufacturer's instructions. Patient immunapheresis material was added to the corresponding column and incubated for 30 min at 4°C. Afterwards, the column was washed with PBS until OD_{280} of the flow through was lower than 0.01, and the bound antibody was eluted in glycine buffer (pH 2.8). The eluate was adjusted to pH 7 using 1M tris base solution (pH 9.0).

HaCaT cells (34) were grown to confluency in 48-well plates in keratinocyte growth medium-2 (KGM-2) (PromoCell GmbH, Heidelberg, Germany) supplemented with medium supplement and calcium chloride solution provided by the supplier at 37°, 5% CO_2. After confluence was reached, cells were incubated with different PI3Ki for 5 min. After incubation, 4 0µg/ml of EBA antibody solutions was added to the designated wells for 24 h. The following controls were included (antibody only, antibody/vehicle, normal human IgG only (Intratect 50 g/L, Biotest AG, Dreieich, Germany, the IgG was re-buffered before use to PBS using Amicon® Ultra-15 Centrifugal Filters), and normal human IgG/vehicle. Cells from two different passages were used and EBA-IgG from 2 donors each, leading to 4 data points.

The C5a content of the supernatants was measured using the Human Complement Component C5a DuoSet ELISA (R&D Systems, Minneapolis, USA) according to manufacturer instructions, with changes to detection using TMB One solution (Promega Corporation, Wisconsin, USA) and 0.9 M H_2SO_4 to stop the color reaction. IL-8 release was measured using the ELISA MAX™ human IL-8 standard set (Biolegend, San Diego, CA, USA) according to manufacturer instructions.

PamGene Measurement

Kinase activity profiles were determined using the PamChip® 4 protein tyrosine (PTK) peptide microarray system from PamGene International B.V. (BJ's-Hertogenbosch, The Netherlands). Human PMNs were diluted to a final concentration of 6.7×10^6 cells/ml in RPMI 1640 (without phenol red) containing 5% FCS and pre-incubated with a final concentration of 5 µM AS-604850, 1 µM alpelisib, 10 µM TGX or vehicle (RPMI 1640 without phenol red/ 5%FCS, 0.1% DMSO) for 5 min (RT). These concentrations were selected as they led to a 50% reduction of ROS release in IC-stimulated PMNs. Flat bottom 6-well-plates were coated with immune complexes consisting of human COL7E-F antigen at a final concentration of 10 µg/ml and anti-human COL7 IgG1 antibody at a final concentration of 2 µg/ml (29), and 10^7 PMNs/1.5 ml per well (containing the respective inhibitors or vehicle) were added. The plates were incubated at 37%, 5% CO_2 for 0, 2, 8, or 15 min, respectively. After incubation, the plates were placed on ice immediately, and the cell solution from each well was transferred to a separate 15 ml tube. Each well was also flushed with 1 ml of ice cold DPBS to detach cells adhered to the plate surface. The cell solutions were centrifuged for 5 min at 700 × g (4°C). The supernatant was discarded, and the cell pellet used to prepare lysates as described in the protocol provided by PamGene (Protocol #1160 for preparation of Lysates of Cell Lines or Purified Cells) (100 µl of lysis buffer per pellet). Protein concentrations in the lysates (above the recommended level >0.5 µg/µL) were determined using the Pierce BCA Protein Assay Kit (Waltham, MA, USA) according to the manufacturer's instructions (microplate procedure).

The Serine-Threonine Kinase (STK) and Protein Tyrosine Kinase (PTK) microarray assays were performed according to the manufacturer's instructions. Samples originating from the same donor and experimental setup ($n = 3$) were placed on the same microarray chip to enable more accurate comparisons. A FITC-conjugated anti-phosphotyrosine antibody was used for visualization during and after the pumping of lysates through the three-dimensional surface of the array. The capture of substrate phosphorylation signals was enabled by a computer-controlled CCD camera and measured repeatedly during a 1-h kinetic protocol using the Evolve software (PamGene International B.V.). The 1-h kinetic protocol showed a linear increase in the signal intensity for the majority of the peptide substrates, indicating that kinome profiling was run without methodological complications. The analysis of the images was performed using the BioNavigator Software (Ver. 6.3), with the predesigned protocols "STK Image Analysis," "PTK Image Analysis," "STK Basic Processing," and "PTK Basic Processing." Additional applications used were "Fit and apply a combat model", "STK upstream kinase analysis," and "PTK upstream kinase analysis." After visual check and quality control, endpoint signal intensities minus background signals were calculated by BioNavigator for each spot representing each kinase peptide substrate per array. Subsequently, the data were $\log_2$-transformed before mean replicate signal intensity within each experiment was calculated for each peptide substrate. A kinase was considered to be modulated (either activated or inhibited) if it had a mean specificity score (= negative decadic logarithm of the likelihood of obtaining a higher difference between the groups when assigning peptides to kinases randomly) of 1 ($p = 0.1$) and a significance score (= likelihood of obtaining a higher difference for random assignment of values to treatment- and control groups) of 0.5 ($p = 0.32$). To detect changes caused by the tested compounds, samples for each timepoint and compound were compared with the control samples for the same timepoint. To ascertain the impact of the stimulation itself, control samples from before stimulation were compared to control samples at

each timepoint (2, 8, and 15 min). The mean kinase statistic (= calculated by averaging the difference between the signal intensity of a sample and its control value, normalized against a pooled estimate of the standard deviation in each sample, for each peptide assigned to a specific kinase) was used for further analysis:

Kinome Trees were created using Coral (35). Venn diagram analyses were performed in Venny2.0 (https://bioinfogp.cnb.csic.es/tools/venny/). Heatmaps were created using in R studio (Ver. 1.3.1093) the heatmap package ("pheatmap" Ver. 1.0.12). Enrichment analysis was performed using Webgestalt Gene Set Enrichment Analysis (36). Settings were as follows: minimum number of genes for a category: 3, maximum: 1000, Significance Level: Top 10. Number of Permutations: 1000. Pathway graphs were created using KEGG Mapper (Ver. 4.2) and sourced from KEGG (Release 95.2) or STRING database (37).

Induction of Experimental EBA in Mice and Treatment Protocol

Rabbits were immunized with a fragment of murine type VII collagen NC1 ($mCOL7^C$) in CFA/IFA by Eurogentec, Köln, Germany. Specific anti-$mCOL7^C$ IgG from immune serum was isolated as previously described (26, 31, 38). Mice were injected in the ear base once with 30–100 μg of specific rabbit anti-mCOL7 IgG per mouse. The respective dose to induce a disease score of 25–50% of the affected ear surface area was determined in advance. PI3K inhibitors were administered orally (**Supplementary Table 1**), or topically applied to the ear 1 day prior to the initial anti-$mCOL7^C$ IgG injection and was performed every day (in total 4 times). The ear thickness measurement, using a Mitutoyo 7301 dial thickness gauge (Neuss, Germany), and scoring was performed by a blinded person. For anesthesia, a mixture of 15 μg/g body weight Xylazin and 100 μg/g body weight Ketamin was injected i.p. into mice (26, 39).

Histopathology and Direct Immunofluorescence Staining

Biopsies of lesional and perilesional skin were obtained on day 3 of the antibody transfer-induced EBA and prepared for examination by histopathology and immunofluorescence (IF) microscopy, as described previously (26, 31, 38). In brief, the biopsies collected from mice were fixed in 4% PBS-buffered formalin, and subsequently, sections of paraffin-embedded tissues were stained with hematoxylin and eosin. IgG deposits were detected by direct IF microscopy in frozen sections prepared from tissue biopsies using fluorescein isothiocyanate (FITC)-labeled antibodies specific to rabbit IgG (Dako, Glostrup, Denmark) as previously described (40).

Statistical Analysis

Unless otherwise noted, data are presented as the mean ± SD or Tukey's box-and-whisker plots; the dots represent actual results for each sample. For comparisons of more than 2 groups, ANOVA was used. For equally distributed data, one-way ANOVA followed by a Bonferroni *t*-test for multiple comparisons was used; if the data were non-parametric, ANOVA on ranks (Kruskal-Wallis) was applied followed by a Bonferroni *t*-test for multiple comparisons. In all tests, $p < 0.05$ was considered indicative of significance. All statistical analyses were performed using SigmaPlot 13.0 (Systat Software, Erkrath, Germany).

For the *in vivo* studies a sample size calculation was performed. Assuming a minimal detectable difference in means of 0.5, a standard deviation of 0.25, and aiming at a power of 0.8 and an α of 0.05, 8 mice per group have to be included when using ANOVA with an appropriate post-test. Hence, 8 mice per group were included in the *in vivo* studies.

RESULTS

IL-8-Induced Chemotaxis of PMNs Is Mainly Impaired by PI3Ki Selective for the β-, γ-, and δ-Isoforms

The directed chemotaxis of polymorphonuclear leukocytes (PMNs) to sites of local inflammation is an early-step in and a key prerequisite for their (patho)-physiological function in EBA (41). Within this multi-step process, cytokines, such as IL-8, are required to facilitate neutrophil extravasation, as well as their activation (42–44). We thus evaluated the impact of inhibition of the selected PI3Ki on IL8-induced PMN chemotaxis. All investigated inhibitors reduced IL-8-induced chemotaxis. PI3K inhibitors mainly selective for PI3Kδ- (AMG319 and IC87114) as well as PI3Kγ-selective (AS-604850 and AMG319) and PI3Kβ-selective inhibitors (TGX-221) inhibited chemotaxis at concentrations within the described IC_{50} values (**Figure 1**). Of note, the pan-PI3K inhibitor GDC-0941 had the most pronounced inhibitory effects on IL-8-induced neutrophil migration, with an efficacy at 0.1 nM. Also, PI3Kα-selective inhibitors (HS-173 and alpelisib) reduced chemotaxis at concentrations above the known IC_{50} values.

PMN Adhesion and Spreading on ICs Are Predominantly Impaired by PI3Kδ- and PI3Kβ-Selective Inhibitors

In addition to ROS release, FcγR-dependent binding of neutrophils also induces their β2 integrin-dependent spreading and adhesion to IC-coated surfaces. This creates an enclosed space between the neutrophils and the target tissue, allowing a directional release of proteases and ROS. If neutrophil adhesion to IC-coated surfaces is blocked, the tissue is protected from the detrimental effects of proteases and ROS (30). We hence evaluated the impact of the PI3Ki on PMN spreading on IC-coated surfaces. Interestingly, PI3K inhibition had only marginal effects on IC-induced spreading and adhesion. Mostly, inhibitors with a PI3Kδ- and PI3Kβ-selectivity reduced PMN spreading (IC-87114, TGX-221, and AMG319, **Figure 2**). As AMG319 inhibits also p110γ, the effects on spreading could be also partially mediated by PI3Kγ. Yet, the second PI3Kγ-selective inhibitor (AS-604850) did not have an effect on IC-induced spreading of PMNs within the investigated concentration range. Furthermore, a statistically significant inhibition was observed for the p110α inhibitor HS-173 at concentrations of 0.1 and 1.0 μM. HS-173, GCD-0941 and alpelisib inhibited neutrophil

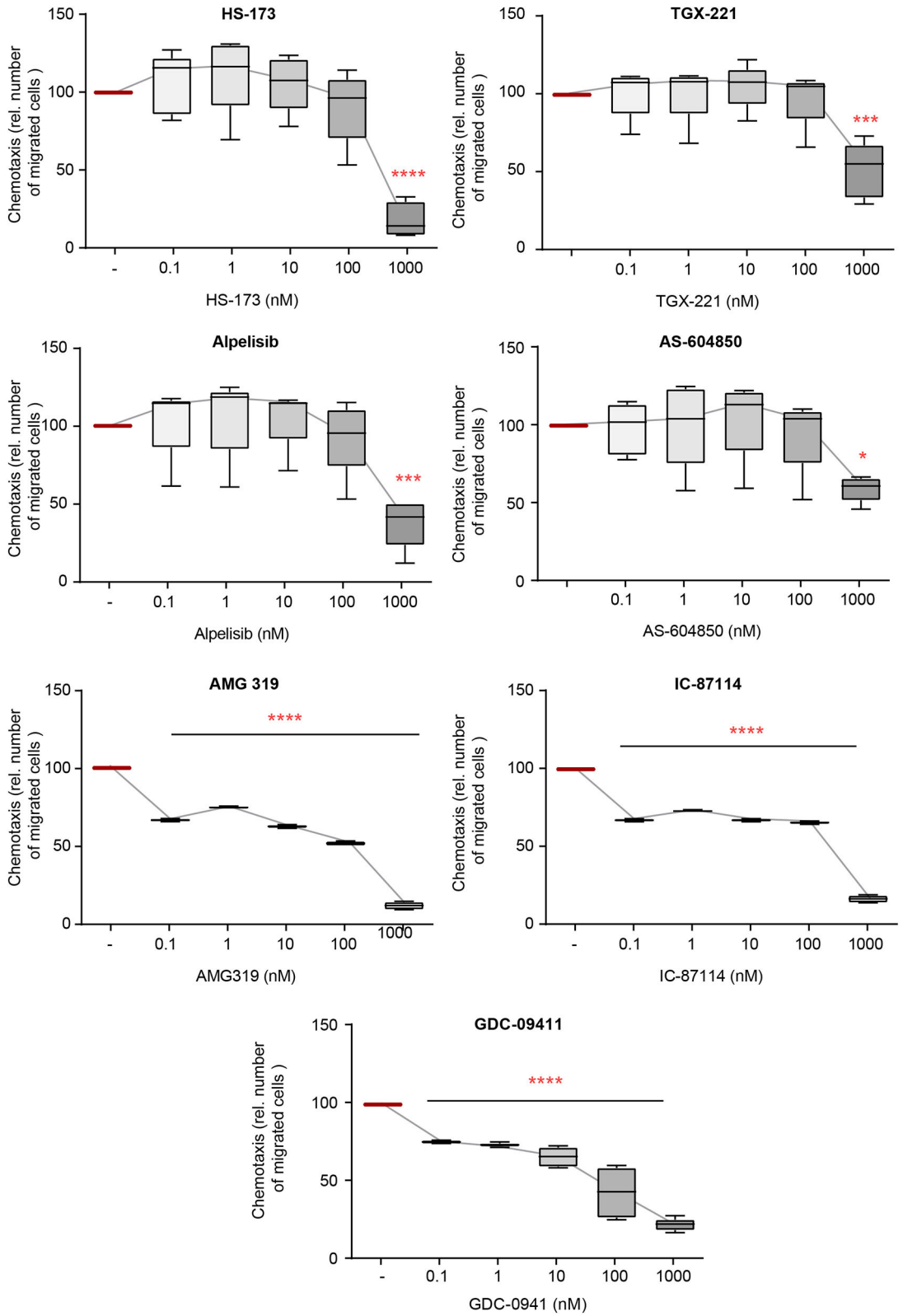

FIGURE 1 | IL-8-induced chemotaxis from PMNs depends on PI3K. Chemotaxis of PMNs was induced by IL-8 in the presence of either one of the PI3K isoform-selective inhibitors. The attracted cell number during a time period of 60 min is shown. Data were normalized to positive control (chemotaxis induced by IL-8 in the presence of solvent). Data are shown as Tukey's box-and-whisker plots. $n = 5$. ANOVA on ranks (Kruskal-Wallis) was applied followed by a Bonferroni t-test for multiple comparisons. $^{*}p < 0.05$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

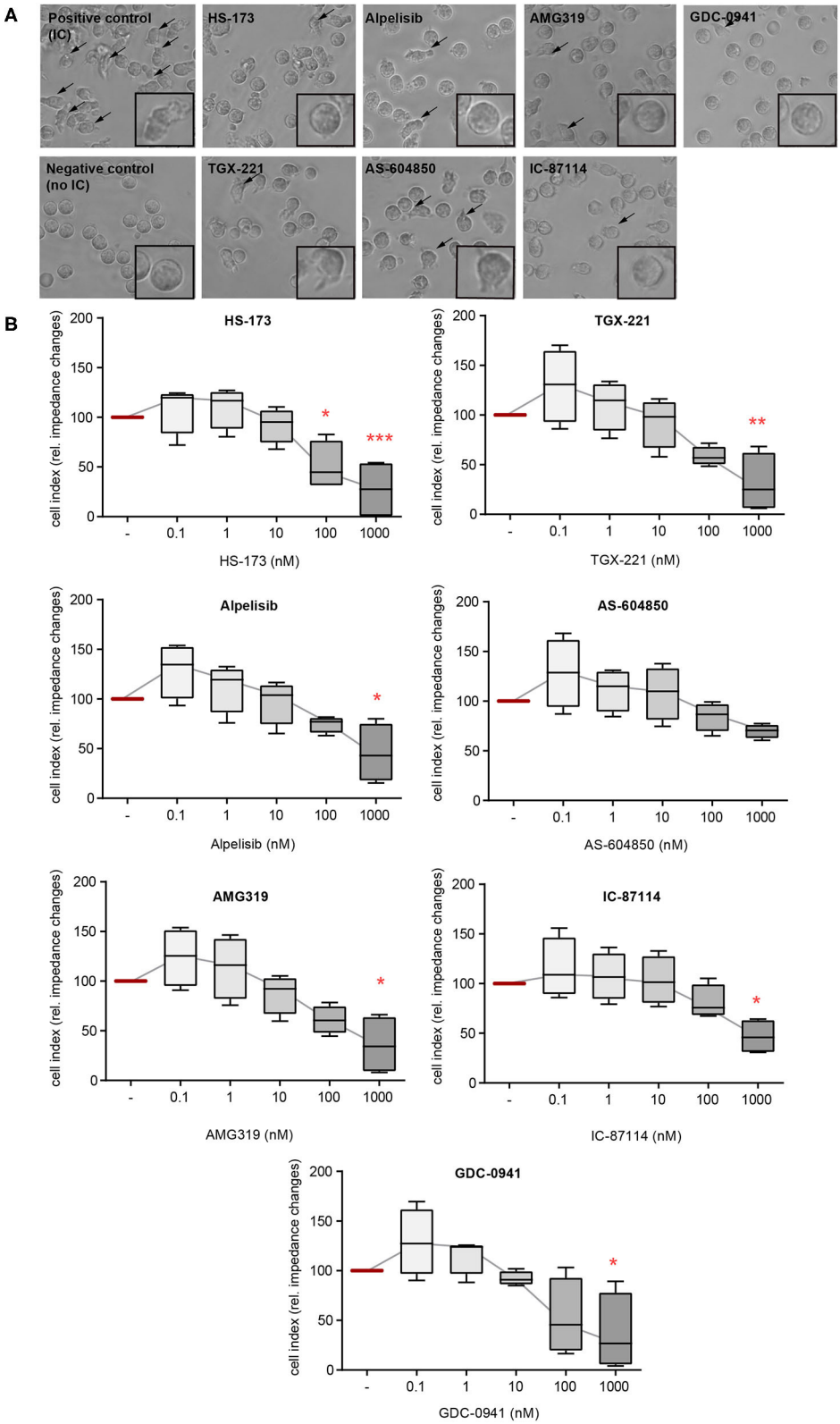

FIGURE 2 | IC-induced spreading of PMNs mainly depends on PI3Kδ. Freshly isolated human blood PMNs were activated with immobilized ICs in the presence of either one of the PI3K isoform-selective inhibitors. **(A)** Microscopic appearance of PMNs 2 h after IC-stimulation. IC-stimulated PMNs show clear spreading on surface (arrows). The highest concentration of all inhibitors (1 μM) reduced the adhesion of PMNs. **(B)** Spreading was monitored live for 2 h and the area under curve (AUC) was analyzed. Data are shown as Tukey's box-and-whisker plots. $n = 4$. ANOVA on ranks (Kruskal-Wallis) was applied followed by a Bonferroni *t*-test for multiple comparisons. Data were normalized to IC-stimulated cells. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

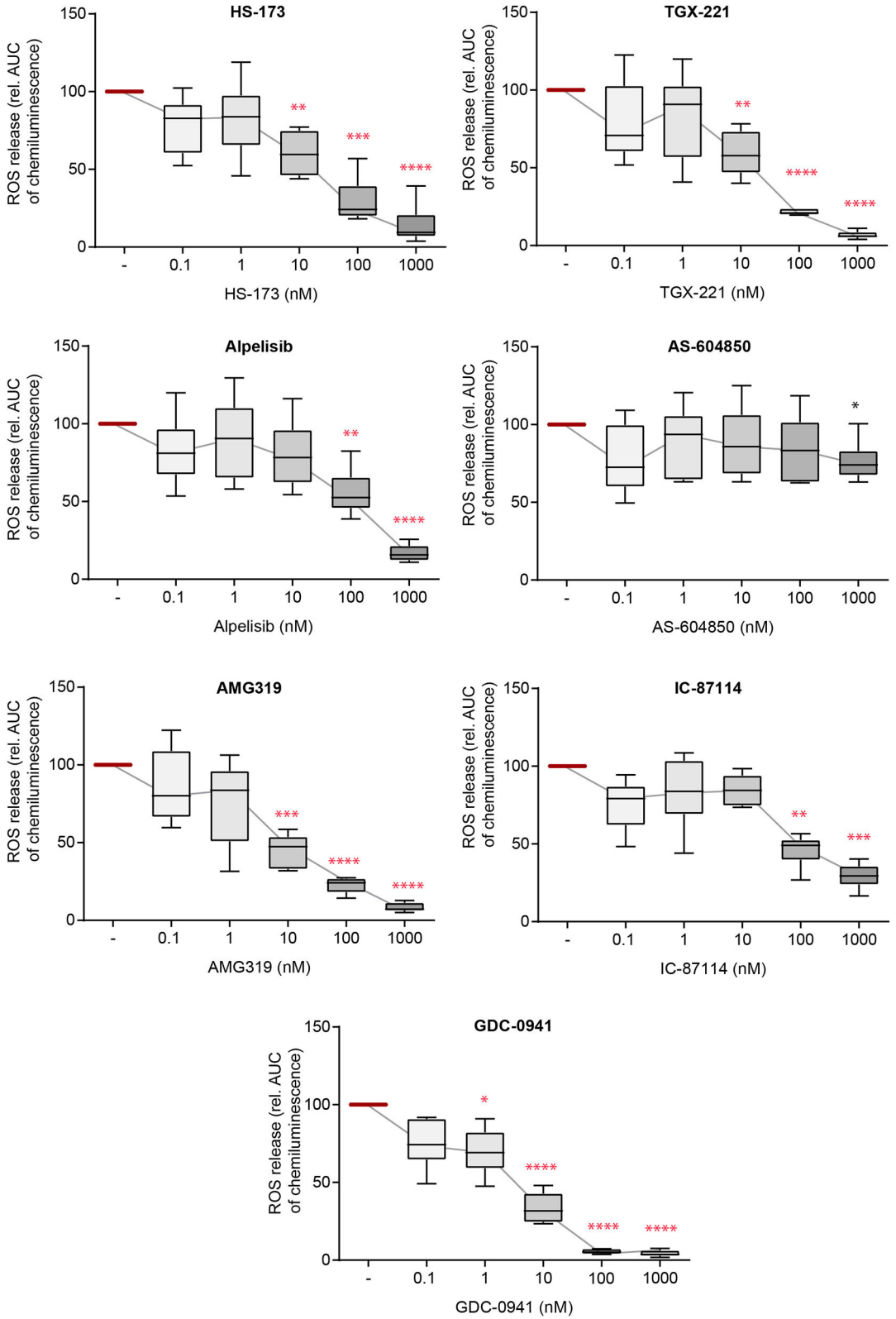

FIGURE 3 | Immune complex (IC)-induced reactive oxygen species (ROS) release from PMNs depends on different PI3K isoforms. Human PMNs were activated with immobilized ICs in the presence of either one of the PI3K isoform-selective inhibitors. Release of ROS was tracked for 2 h and the area under curve (AUC) was calculated. Data were normalized to positive control (IC-stimulated PMNs with solvent). Data are shown as Tukey's box-and-whisker plots. $n = 6$. ANOVA on ranks (Kruskal-Wallis) was applied followed by a Bonferroni *t*-test for multiple comparisons. Data were normalized to stimulated cells. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

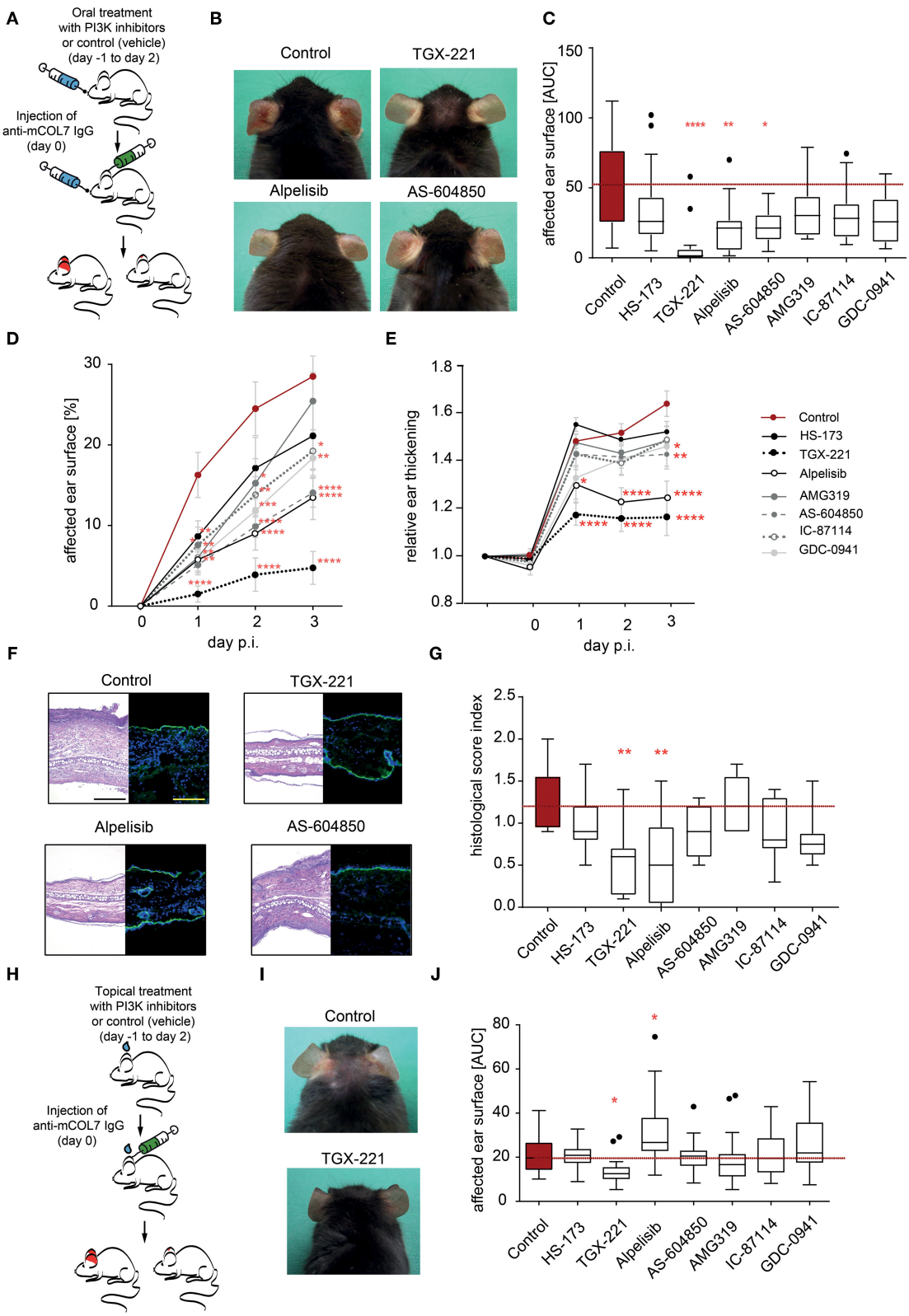

FIGURE 4 | Treatment with TGX-221, alpelisib or AS-604850 impairs induction of experimental EBA. **(A)** Schematic illustration of experimental work flow. Anti-mCOL7 IgG was injected into the ears of C57BL/6J mice and the mice were orally treated with the indicated PI3K isoform selective inhibitors. **(B)** Representative clinical images of the mice on day 3. **(C)** The cumulative affected ear surface area (AUC) in mice treated with solvent or the indicated PI3K inhibitors. Data are shown

(Continued)

FIGURE 4 | as Tukey's box-and-whisker plots. ANOVA on ranks (Kruskal-Wallis) was applied followed by a Bonferroni t-test for multiple comparisons. **(D)** Percentage of ear surface area affected by EBA skin lesions at days 0–3 of the same experiment. Data are shown as mean ± SD. Two way-ANOVA with Bonferroni post test. **(E)** Relative ear swelling at days 0–3 of the same experiment. Data are normalized to day 0 of the experiment and shown as mean ± SD. Two way-ANOVA with Bonferroni post test. **(F)** Left panel: Representative H&E stained ear skin at day 3 after IgG-injection. Right panel: Representative direct IF microscopy of a skin biopsy, stained for anti-rabbit IgG (green) and nuclei (DAPI, blue). **(G)** The cumulative histological score index (AUC of skin infiltration, epidermal thickening and split formation at the DEJ) was analyzed in ear skin at day 3 of the experiment. Data are shown as Tukey's box-and-whisker plots. ANOVA on ranks (Kruskal–Wallis) was applied followed by a Bonferroni t-test for multiple comparisons. Data in panels **(C–E,G)** is based on 8 mice/group. **(H)** Schematic illustration of experimental work flow for topical treatment with PI3K isoform-selective inhibitors. Anti-mCOL7 IgG was injected into the ears of C57BL/6J mice and the mice were topically treated daily with the indicated PI3K isoform-selective inhibitors. **(I)** Representative clinical images on day 3 after topical treatment with solvent or TGX-221. **(J)** Cumulative affected ear surface area (AUC of percentage of day 0–3) in mice with topical PI3K inhibitor treatment. Data are shown as Tukey's box-and-whisker plots. ANOVA on ranks (Kruskal-Wallis) was applied followed by a Bonferroni t-test for multiple comparisons. Data in panels **(I,J)** is based on 8 mice/group. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

spreading and adhesion only at the 1 μM dose, which are relative high doses (**Figure 2**).

IC-Induced ROS Release From PMNs Is Sensitive to Blockade of All PI3K Isoforms

Activation of neutrophils and subsequent ROS release by FcγR-dependent binding to IC initiates local inflammation across many autoantibody-mediated diseases, including EBA (12, 45, 46). Herein, we thus used IC to activate PMN and evaluated the impact of selective PI3Ki on IC-induced ROS release. Here, all inhibitors, with the exception of PI3Kα subclass-selective inhibitors, impaired ROS release from IC-activated PMNs at doses close to the reported IC_{50} (**Figure 3**). The pan-PI3K inhibitor GDC-0941 had the most profound inhibitory effects, with efficacy at 1.0 nM. All effects were independent of any cytotoxic effects on neutrophils as none of the inhibitors effected Annexin V/propidium iodide staining in IC-stimulated neutrophils (**Supplementary Figure 1**).

Selective Pharmacological Inhibition of PI3K Did Not Influence C5a and IL-8 Release From Keratinocytes

So far, we had demonstrated a selective contribution of PI3K to distinct neutrophil functions *in vitro*. To evaluate possible effects on other disease-relevant cell types, we stimulated human keratinocytes with EBA-IgG. We hence evaluated the impact of the PI3Ki on C5a and IL-8 release after 24 h of stimulation (**Supplementary Figure 2**) as complement activation and IL-8 release are key events in the effector phase of EBA and other pemphigoid diseases (21, 47–49). Compared to normal human IgG, EBA-IgG significantly increased IL-8 and C5a concentrations in the supernatants of HaCaT cells. Yet, none of the analyzed inhibitors had an inhibitory effect on EBA-IgG activated C5a release. Of note, after stimulation with EBA-IgG, several inhibitors (TGX-221, alpelisib, and IC-87115) showed a slight tendency even to increase the C5a release if used in low concentrations. Nevertheless, higher concentrations of the inhibitors did not affect C5a release. Interestingly, release of IL-8 was slightly inhibited by alpelisib at concentrations above the known IC_{50} values (1 μM) whereas none of the other inhibitors had any effect.

Selective Pharmacological Inhibition of PI3K Reduced Inflammation in Experimental EBA

So far, we had demonstrated a selective contribution of PI3K to distinct neutrophil functions *in vitro*. Pathogenesis of inflammation in autoantibody-induced and neutrophil-dependent diseases, is, however, a multistep process that involves all (if not more) of the above tested steps (12). Furthermore, *in vivo* pharmacokinetics of the PI3Ki are also key determinants of their potential disease-modifying activity. Thus, prediction which PI3K inhibitor has anti-inflammatory effects in pre-clinical models of EBA is challenging. To address this, we used the mouse model of antibody transfer-induced EBA, in which disease manifestation depends on the presence of COL7 (auto)antibodies and a subsequent FcγR-dependent neutrophil activation (21). In a first set of experiments, mice, in which EBA was induced by anti-COL7-IgG injection, were orally treated with PI3K-selective inhibitors in a concentration that was recently shown to be effective known effective for other applications (50–56) (**Figure 4**). The p110α-selective inhibitor alpelisib and the p110γ-selective inhibitor AS-604850 showed impaired disease induction (ear thickness increase and affected ear surface area) by 50–60% (**Figures 4D,E**). Treatment with the p110β-selective inhibitor TGX-221 almost completely abolished the pathogenic activity of the anti-mCOL7 antibodies. A minor effect was observed for GDC-0941 and IC-87114 as visible by a reduced affected surface area (**Figure 4D**). All other tested inhibitors (HS-173 and AMG319) had no effect on clinical disease manifestation at the selected doses (**Figures 4A–G**).

Topical Application of the PI3Kβ-Selective Inhibitor TGX-221 Impairs Induction of Experimental EBA

As systemic PI3K inhibition in humans may be associated with a relative high number of (serious) adverse events (27, 57), we next evaluated if topical application of the PI3Ki has an impact on disease manifestation in experimental EBA. Of the seven inhibitors, the p110β-selective inhibitor TGX-221 again profoundly impaired the induction of skin inflammation (**Figures 4H–J**). By contrast, all other PI3Ki did not reduce clinical disease manifestation in experimental EBA at selected concentration. During all experiments in the pre-clinical EBA mouse model, neither oral nor topical treatment with one of the PI3K inhibitors led to an increased suffering

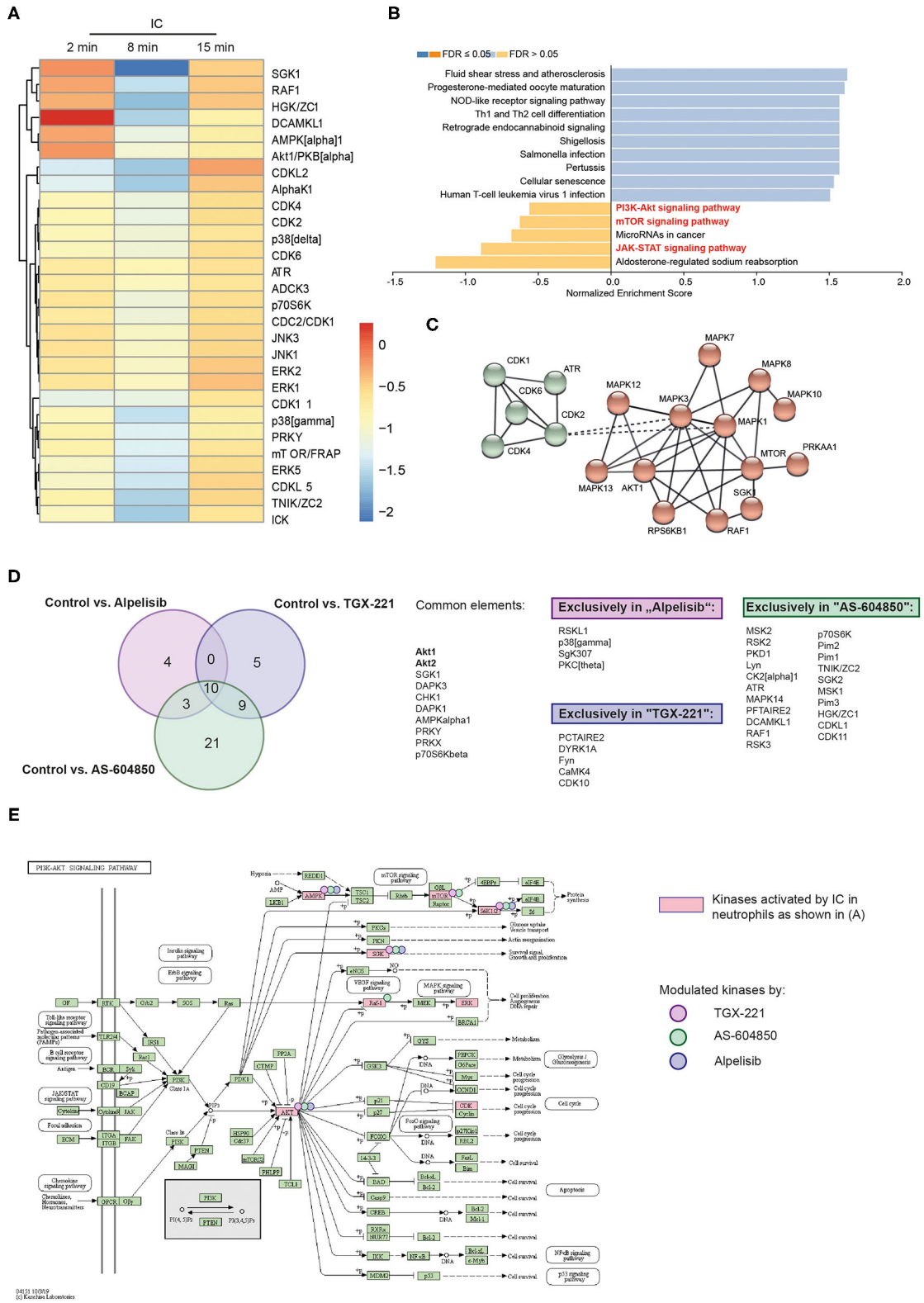

FIGURE 5 | Unique impact of different PI3K inhibitors on downstream kinase activation. Freshly isolated human blood PMNs were activated with IC for 0, 2, 8, or 15 min. Cells were lysed and the PTK and STK activity was measured by PamGene. **(A)** Heatmap of mean kinase statistic in comparison to unstimulated cells (0 min). Blue: Kinase activity decreased/Red: Kinase activity increased. **(B)** Enriched KEGG pathways (as shown by Webgestalt) after IC-stimulation. **(C)** String network for

(Continued)

FIGURE 5 | IC-stimulated kinases (MCL clustering, inflation parameter 3) **(D)** Venn diagram and summary of the common and uniquely regulated kinase activities after use of three PI3K isoform-selective inhibitors that were effective in experimental EBA (alpelisib, AS-604850 and TGX-221). The comparison was performed for all time points with direct comparison to the kinase activity of ICs activated PMNs treated with solvent. **(E)** Influence of PI3K inhibition on PI3K/Akt and mTOR pathways, kinases regulated by ICs (cumulative stimulation for 2, 8, and 15 min using PamGene) are shown in bright red. Kinases, that are regulated by the respective kinase inhibitors are marked with a reddish dot (TGX-221), green dot (AS-604850), and purple dot (alpelisib). Data is based on 3 replicates per group.

(weight reduction, general condition and behavior) in the mice (**Supplementary Tables 2, 3**).

Kinome Analysis of Identified Unique Downstream Signaling Pathways of the Different, Disease-Modifying PI3K-Selective Inhibitors

At this point, using the EBA mouse model, we had demonstrated that TGX-221, alpelisib or AS-604850 impaired induction of experimental EBA. To obtain more detailed insights into the orchestration of IC-induced signal transduction pathways selectively inhibited by these 3 therapeutically promising drugs, we performed a multiplex kinase activity profiling using PamGene (58). We incubated human PMNs with PI3Ki (alpelisib, AS-604850 or TGX-221) for 5 min and subsequently stimulated PMNs with IC for 0, 2, 8, or 15 min. As expected, IC-stimulation of PMNs induced, among others, the PI3K/Akt, JAK/STAT and mTOR pathways (**Figures 5A,B** and **Supplementary Table 4**). In addition to these known pathways, we found a strong activity of several cyclin-dependent kinases (CDKs, **Figure 5C**). These are enriched in the KEGG pathway for "cellular senescence" (**Supplementary Figure 3**). Other relevant pathways that were regulated by IC-stimulation are summarized in **Supplementary Figures 3, 4** (mTOR signaling, FoxO signaling and FcRγ-mediated phagocytosis).

To obtain more detailed insights into the mode of action of the effective inhibitors, we compared the kinase activity in PMNs after PI3K inhibition with the corresponding control time points (**Figure 5D**). Inhibition of PI3K by TGX-221 (**Supplementary Figure 5**), alpelisib (**Supplementary Figure 6**) or AS-604850 (**Supplementary Figure 7**) led to a decreased activity of Akt1/2 and the downstream kinases SGK, p70S6Kbeta (S6K1) and AMPK (**Figures 5D,E**). Interestingly, all 3 inhibitors also had unique effects on several kinases and KEGG pathways that were independent of the known PI3K network (**Figure 5D**). More specifically, inhibition using AS-604850 (**Supplementary Figure 7**) identified a total of 21 kinases that were exclusively de- or increased in their activity, indicating additional effects on different targets. By contrast, only 5 unique kinases were detected in TGX-221-treated PMN and 4 unique kinases in alpelisib treated PMNs (**Figure 5** and **Supplementary Tables 5–7**).

DISCUSSION

We here systematically investigated the impact of seven PI3Ki with different isoform-selectivity on neutrophil functions *in vitro*, as well as their potential to reduce autoantibody-induced, neutrophil-driven inflammation in preclinical models of EBA. We demonstrate that the PI3Kβ-selective TGX-221 has the most pronounced disease-modifying activity, especially when applied topically. Of note, this superior *in vivo* effect of TGX-221 could not be derived from the *in vitro* characterization of the PI3Ki evaluated herein. More specifically, TGX-221 impaired IL-8-induced chemotaxis only at relatively high concentrations, while AMG 319 and IC-87114 dose-dependently and at quite low concentrations, reduced PMN chemotaxis. PMN spreading to IC, as well as IC-induced ROS release from PMN was equally well inhibited by HS-173, TGX-221, as well as several more PI3Ki in the IC-induced ROS release. Thus, *in vivo* disease models are required to demonstrate efficacy.

Hence, while this and previous (16, 18) reports demonstrate the fundamental role of PI3Kβ and PI3Kδ in the pathogenesis of skin inflammation in EBA, our data presented here does not rule out that other class I PI3K isoforms contribute to EBA pathogenesis—especially PI3Kα, one PI3Ki selective for this isoform (alpelisib) impaired induction of experimental EBA. Interestingly, alpelisib was blocking chemotaxis and ROS release only if used at rather high concentrations but in contrast reduced IL-8 release from human keratinocytes. Still, the kinase activation profiles indicate a specific inhibition of the PI3K pathway by alpelisib. In addition to these *in vitro* findings, the *in vivo* pharmacokinetics of a given drug are crucial for its activity. For alpelisib, over 50% are absorbed, with a $T_{(max)}$ of 2 h and an elimination half-life from plasma of 13.7 h (59) which indicates a good bioavailability that could contribute to the effectivity of alpelisib in murine experimental EBA.

Dissection of the contribution of different PI3K isoforms to EBA pathogenesis, would require the use of PI3K isoform-deficient mice (1, 2, 60–62). We here, however, specifically focused on pharmacological PI3K inhibition allowing a better clinical translation.

In the pathogenesis of the inflammatory type of EBA, PMNs exert their pathogenic effects in a stepwise manner that is initiated by attracting them into the tissue (chemotaxis) and ultimately results in the release of pro-inflammatory substances, such as ROS (**Figure 6**). Extravasation of PMNs to sites of inflammation is an early key step driving EBA (63, 64). Within this multi-step process, cytokines, such as IL-8, are required to facilitate neutrophil chemotaxis, as well as activation (42–44). We here demonstrate that PI3Kγ- and PI3Kδ- selective inhibitors block PMN chemotaxis. By contrast, selective inhibitors of PI3Kα have no major impact on IL-8-induced PMN migration. These findings support earlier notions demonstrating that in fMLP-stimulated neutrophils, the PI3Kδ inhibitor IC-87114 affected polarized morphology of neutrophils, PIP_3 production and chemotaxis, but did not block F-actin synthesis or neutrophil adhesion (65). Regarding PI3Kγ, G-protein-coupled receptor

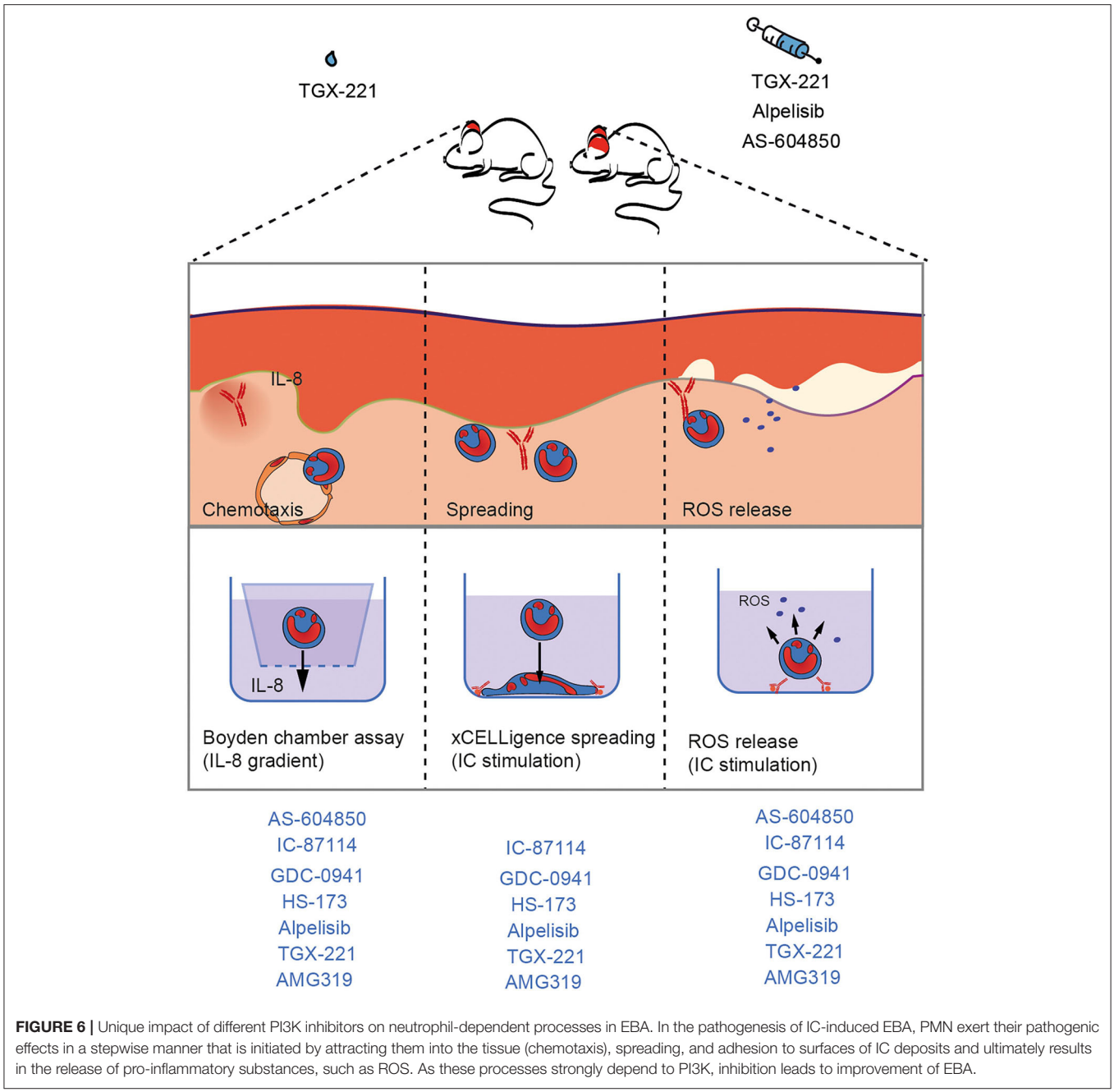

FIGURE 6 | Unique impact of different PI3K inhibitors on neutrophil-dependent processes in EBA. In the pathogenesis of IC-induced EBA, PMN exert their pathogenic effects in a stepwise manner that is initiated by attracting them into the tissue (chemotaxis), spreading, and adhesion to surfaces of IC deposits and ultimately results in the release of pro-inflammatory substances, such as ROS. As these processes strongly depend to PI3K, inhibition leads to improvement of EBA.

(GPCR) stimulation of PI3Kγ induced PMN migration (and the oxidative burst) (66). Therefore, both PI3Kγ and δ seem to be important to mediate PMN chemotaxis across several stimuli.

By creating a protected space between the neutrophil and the target tissue, neutrophil spreading and adhesion to surfaces of IC deposits is a prerequisite for autoantibody-induced tissue damage (30). This process is regulated by PI3K activation (67, 68). Previous data demonstrated that the pan-PI3K inhibitor wortmannin or the PI3Kα/β/δ inhibitor LY294002 impaired neutrophil binding to ICAM-1 after fMLP stimulation (69). Other data on the PI3K isoforms involved in these processes is sparse. PI3Kγ has been shown to mediate the transition from rolling to firm adhesion, a process depending on β2 integrins (70). We here show that β2 integrin-dependent neutrophil adhesion to immobilized IC mostly depends on PI3Kγ /δ.

Regarding ROS release from IC-activated neutrophils, we here show that this requires PI3Kβ, γ, and δ. These findings are consisted with published data for PI3Kβ and δ (16, 18), which may even act synergistically in this context (16). Interestingly, while important for TNF/fMLP-induced ROS release from neutrophils (19), as shown here, PI3Kγ-selective inhibition only has a marginal impact on IC-induced ROS release. In line with this, pan-PI3K inhibition completely abolished the IC-induced ROS release.

All of the above neutrophil functions contribute to local inflammation in EBA (21). Yet, the *in vitro* inhibitory effects of PI3K isoform-selective drugs do not allow to predict their impact on the complex disease pathogenesis *in vivo*. To investigate if inhibition of specific PI3K isoforms has an impact on *in vivo* inflammation provoked by tissue-bound IC, we used the antibody transfer model of EBA. We specifically selected a skin inflammation mouse model because topical application, which presumably leads to less adverse events, is possible in chronic inflammatory skin diseases. Out of 7 tested PI3K inhibitors, AS-604850 (PI3Kγ-selective), alpelisib (PI3Kα-selective) and TGX-221 (PI3Kß-selective) showed a significant inhibition of ear thickening and led to a more than 50% reduction of the affected ear surface area. One of these inhibitors, TGX-221 was even effective if applied topically. Given that EBA is notoriously difficult to treat, achieving remissions after 9 month of systemic immunosuppressive treatment (23), addition of a (preferably topically applied) PI3Ki could potentially lead to a more rapid induction of remission.

Previously, we had documented that induction of skin inflammation is hampered in PI3Kβ-deficient mice or in mice treated with a PI3Kδ-selective inhibitor (16, 18). Overall, these data and the results presented herein point toward a non-redundant role of these 2 class I PI3K isoforms.

Next, we here evaluated the impact of the three PI3K isoform-specific inhibitors on downstream signaling cascades, allowing detailed insights into PI3K isoform-specific signaling pathways in human PMNs, which may provide additional and more downstream targets to block IC-induced PMN activation. Unbiased analysis of kinase activity in IC-activated PMN treated with either TGX-221, alpelisib or AS-604850 identified a number of shared downstream kinases like Akt1/2 and the downstream kinases SGK, p70S6Kbeta (S6K1) and AMPK. However, the majority of changes in kinase activity after treatment of PMN with either of the compounds was specific for each inhibitor, and most pronounced for AS-604850.

Collectively, we here defined the differential impact of PI3K isoforms on immune complex-induced neutrophil signaling and function, and identify the PI3Kβ-selective TGX-221 as a potential topical treatment for the inflammatory type of EBA and other autoimmune skin blistering diseases with a similar pathogenesis.

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/s.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by University of Lübeck, Lübeck, Germany, AZ 09-140. The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by Animal Care and Use Committee (Kiel, Germany)—AZ122.5(108/08-15).

AUTHOR CONTRIBUTIONS

RL and KBi: conceptualization and methodology and writing—original draft. HZ, AK, NG, CO, CH, MR, VH, NE, and RV: investigation. KBi and CO: data curation. RL: funding acquisition and project administration. RL, GV, FP, TL, and XY: resources. MB-H, XY, FP, RL, and KBi: supervision. HZ, AK, CO, NG, MR, CH, VH, MB-H, NE, KBo, GV, RV, TL, XY, FP, RL, and KBi: writing—review and editing. 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/fmed.2021.713312/full#supplementary-material

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SOCS Proteins in Immunity, Inflammatory Diseases, and Immune-Related Cancer

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Cytokine signaling represents one of the cornerstones of the immune system, mediating the complex responses required to facilitate appropriate immune cell development and function that supports robust immunity. It is crucial that these signals be tightly regulated, with dysregulation underpinning immune defects, including excessive inflammation, as well as contributing to various immune-related malignancies. A specialized family of proteins called suppressors of cytokine signaling (SOCS) participate in negative feedback regulation of cytokine signaling, ensuring it is appropriately restrained. The eight SOCS proteins identified regulate cytokine and other signaling pathways in unique ways. SOCS1–3 and CISH are most closely involved in the regulation of immune-related signaling, influencing processes such polarization of lymphocytes and the activation of myeloid cells by controlling signaling downstream of essential cytokines such as IL-4, IL-6, and IFN-γ. SOCS protein perturbation disrupts these processes resulting in the development of inflammatory and autoimmune conditions as well as malignancies. As a consequence, SOCS proteins are garnering increased interest as a unique avenue to treat these disorders.

Keywords: SOCS, cytokine, immunity, inflammation, cancer

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CYTOKINE SIGNALING IN IMMUNITY AND ITS REGULATION

Overview

Appropriate immune cell development and function relies on the complex interplay between various cell lineages, much of which is mediated by cytokines. These include the interleukin (IL)-2 family, comprising IL-2, IL-7, IL-9, IL-15, and IL-21, that play important roles in the development of specific lymphoid lineages (1, 2), the IL-3 family, IL-3, IL-5, and granulocyte/macrophage colony-stimulating factor (GM-CSF) (3) and members of the IL-6 family, especially IL-6, IL-10, and granulocyte-CSF (G-CSF) (4), that regulate myeloid lineage development and also impact on inflammation, as well as the interferons (IFNs), that mediate antiviral responses and modulate immune cell development and inflammation. Signaling by cytokines needs to be tightly controlled in order to ensure the immune system is maintained at homeostatic levels, as this could result in the development of inflammatory conditions, increased susceptibility to infectious disease and for pathologies as well as an increased propensity to develop cancer (5–7).

Cytokine Signaling and Its Control

Cytokines bind to cell surface receptors on responsive cells triggering a series of intracellular events that ultimately alter the state of the cell through the stimulation of important genes

(8). The majority of cytokine receptors utilize the Janus kinase (JAK)/Signal transducer and activator of transcription (STAT) pathway in order to transmit signals to the nucleus (**Figure 1**) (6). Mammals possess four JAKs (JAK1, JAK2, JAK3, and TYK2) (9) and seven STATs (STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B, and STAT6) (10), with specific cytokine receptors using a particular combination of these to influence immune cell production and function. In response to cytokine binding to specific receptors, a phosphorylation cascade is initiated resulting in tyrosine phosphorylation of STATs found in the cytoplasm (11). Upon phosphorylation, STATs dimerize through reciprocal interactions, with STAT dimers subsequently entering the nucleus *via* an active process involving importin proteins (12) and GTPases such as Rac1 and Ran (13, 14), allowing them to initiate transcription of target genes (15).

A variety of proteins are involved in the negative regulation of cytokine receptor signaling through the JAK/STAT pathway such as SH2-containing phosphatases (SHPs), protein inhibitors of activated STATs (PIASs) and suppressors of cytokine signaling (SOCS) proteins (16). Both SHP and PIAS proteins are present in the cytosol prior to the onset of signaling. SHPs regulate signaling through mediating dephosphorylation of various pathway components following recruitment *via* their SH2 domain (17). Alternatively, PIASs inhibit the activity of STATs at various levels and thereby dissipate signaling (17). In contrast, SOCS proteins are negative feedback regulators that are induced directly by STAT proteins and in turn act to negatively regulate the JAK/STAT pathway *via* various mechanisms to ensure appropriate signal dissipation (18).

SOCS PROTEINS

There are eight human SOCS proteins, SOCS1–7 and cytokine inducible SH2-containing protein (CISH) (**Figure 2**) (19). All SOCS proteins show conserved structural similarities and mechanisms of action but with unique aspects for different family members (16, 20), with pairs of SOCS proteins showing greater similarity reflecting their evolutionary history (21). Indeed, there is strong conservation of SOCS proteins, with other mammalian species harboring an equivalent complement and other higher vertebrates having homologs for each, with additional duplicates in teleost fish (22). Invertebrates such as Drosophila also possess multiple SOCS proteins that also participate in the regulation of cytokine and other signaling (23), highlighting the importance of these negative regulators within the cytokine receptor/JAK/STAT pathway.

Structure of SOCS Proteins

All SOCS family members contain two conserved regions organized in a signature arrangement comprising a central Src-homology 2 (SH2) domain and a C-terminal SOCS box domain (**Figure 2**) (24). The SH2 domain allows SOCS proteins to bind target substrates through interactions with phosphorylated tyrosine residues (25). However, unlike the canonical SH2 domain found in many signaling proteins, those of SOCS proteins contain an N-terminal α-helical extension, called the extended SH2 domain (ESS) (26). The SOCS box is able to assemble components of an E3 ubiquitin ligase complex *via* two motifs, the Elongin B/C (BC) box and the Cullin (Cul) box (27, 28). The largest variability in SOCS protein structure is observed at the N-terminus (19). The N-terminal domains of SOCS1–3 and CISH are of similar length, but those of SOCS4–7 are considerably longer (29). Specialized motifs within the N-terminal domains have been identified in related SOCS proteins. SOCS1 and SOCS3 contain a unique kinase inhibitory region (KIR) that is able to bind and inhibit JAKs (30), while SOCS3 and CISH contain a PEST motif between the SH2 domain and SOCS box (26, 31), with SOCS4 and SOCS5 having a distinct N-terminal conserved region (NTCR), the exact function of which is yet to be determined (29).

Mechanisms of SOCS Action

SOCS proteins regulate cytokine receptor signaling through several different mechanisms such as competitive binding, targeting proteins for degradation/re-routing, and inhibition of kinase activity, which utilize different combinations of protein domains and motifs (**Table 1**). However, in each case the SH2 domain typically allows SOCS proteins to bind to specific components of the cytokine receptor signaling complex or downstream signaling proteins through interactions with appropriate phosphotyrosine containing motifs (**Figure 3**) (7).

Competitive Binding

SOCS proteins are able to bind to receptor complexes and sterically block binding of other molecules. This is generally through interactions of their SH2 domain to phosphotyrosine motifs to impede binding of other SH2 domain-containing proteins, notably including STATs, thereby inhibiting their activation (**Figure 3A**), but other mechanisms also exist (7).

Protein Targeting

SOCS proteins are also able to target proteins, including the receptor and associated JAK and other signaling proteins (24, 53), through formation of an E3 ubiquitin ligase complex (**Figure 3B**). Elongin BC and Cullin 5 are recruited directly to specific motifs in the SOCS box domain as part of a complex containing Ring box protein 2 (Rbx2) and a ubiquitin conjugating enzyme (E2) (54). Once the E3 ubiquitin ligase complex is assembled, target proteins bound through the SH2 domain undergo ubiquitination, leading to their proteasomal degradation or intracellular re-routing (55).

Inhibition of JAK Activity

SOCS1 and SOCS3 can also directly inhibit the kinase activity of receptor-associated JAK proteins thereby suppressing signal propagation (**Figure 3C**) (56). SOCS1 binds to the JAK proteins directly through its SH2 domain (57), whereas SOCS3 instead binds to the associated receptor (57, 58). In each case the respective KIR motif is then able to act as a pseudo-substrate thereby blocking the substrate-binding groove of the JAK kinase domain to inhibit its activity (57).

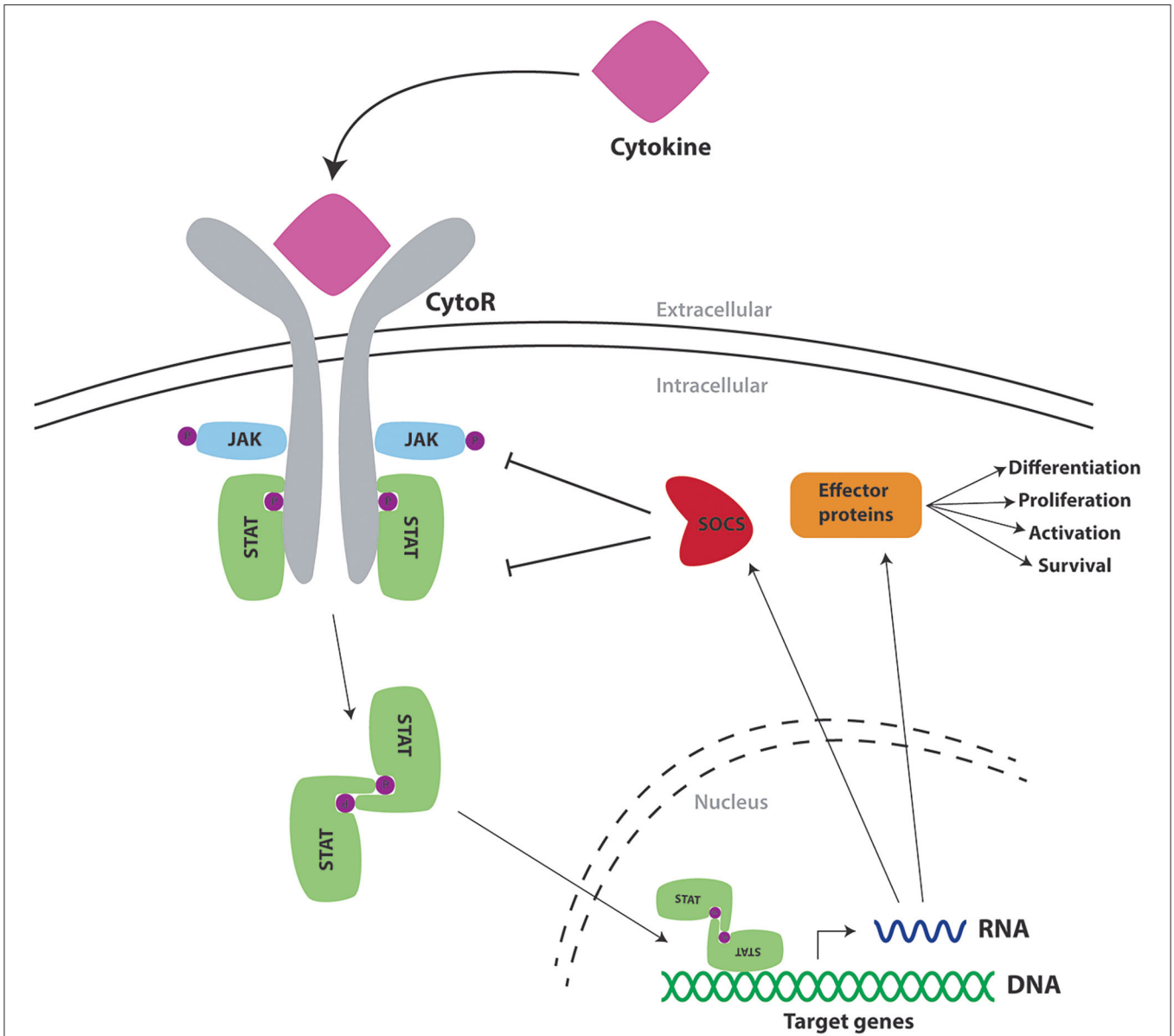

FIGURE 1 | Cytokine signaling via the JAK/STAT pathway and its regulation by SOCS proteins. Schematic representation of a cytokine (pink) binding to the extracellular portion of its cognate cytokine receptor (CytoR—gray), to cause activation of associated intracellular Janus kinases (JAK—blue), which mediate phosphorylation (P—purple) of tyrosine residues found on components of the receptor complex. These form docking sites for signal transducer and activator of transcription proteins (STAT—green), which also undergo phosphorylation to form STAT dimers. These translocate into the nucleus to activate transcription of target genes encoding effector proteins (orange) as well as SOCS proteins (red) that provide negative feedback regulation.

Regulation of SOCS Proteins

SOCS protein levels have been shown to be regulated by multiple layers of control. At the transcriptional level, SOCS genes—particularly *SOCS1*, *SOCS2*, *SOCS3*, and *CISH*—are strongly induced by activated STATs (59), but other transcription factors can additionally mediate this (60). SOCS genes can also repressed by others, as described for *SOCS1* and *SOCS3* by growth factor independence-1 (GFI-1) through promoter binding (61, 62), and can be silenced epigenetically through regulation through methylation of CpG islands or histone deacetylation (63–65). SOCS mRNAs are typically short-lived, with $AU_{2-4}A$ motifs in the 3′UTR linked to stability (31, 66), along with m^6A methylation (67). Furthermore, the 5′UTR of the SOCS1 mRNA has an inhibitory effect on translation mediated by an upstream open reading frame (68), and can also influence translation of different protein isoforms (69). In addition to this, translation is controlled by a myriad of micro-RNAs (miRNAs) that typically bind to sites located in the 3′UTR (70–74). SOCS proteins are also

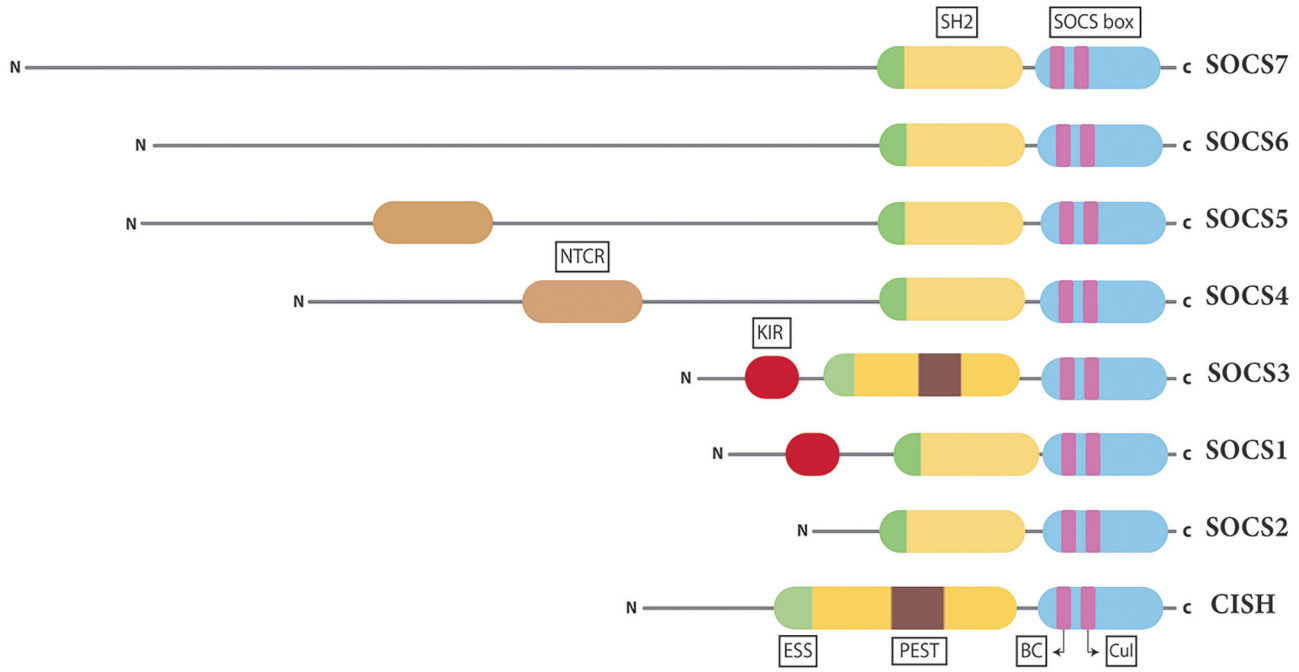

FIGURE 2 | Structure of the SOCS proteins. Schematic representation of the eight mammalian SOCS proteins presented in pairs based on conserved structure and function. Shown are the universal central SH2 domain (yellow), including a unique extra α-helical structure termed the extended SH2 domain (ESS) (green) found in all SOCS proteins and a PEST motif (purple) found in SOCS3 and CISH as well as the C-terminal SOCS box domain (blue), containing Elongin BC (BC) and Cullin (Cul) boxes (pink). In addition, there is a variable N-terminal domain, containing a specialized kinase inhibitory region (KIR) in SOCS1 and SOCS3 (red) and an N-terminal conserved region (NTCR) in SOCS4 and SOCS5 (brown).

relatively unstable, with binding to elongins and phosphorylation of the SOCS box found to influence relative protein stability (75–77). CISH and SOCS3 also possess a PEST motif adjacent to their SH2 domain, which provides an additional mechanism to impact protein stability (26, 31).

SOCS PROTEINS AS REGULATORS OF IMMUNITY AND ITS PERTURBATION IN DISEASE

SOCS proteins, particularly, SOCS1–3 and CISH are closely involved in the regulation of cytokine receptor signaling (19, 78). Since cytokines play a key role in immune cell development and function, SOCS proteins influence multiple aspects of immune system development and function, notably including helper T (Th) cell differentiation (**Figure 4**) and myeloid cell development and function (**Figure 5**). They are also implicated in a range of inflammatory and autoimmune diseases as well as immune-related cancers.

SOCS1

SOCS1 is induced by a wide range of cytokines, including IFN-γ *via* STAT1 and those that utilize the IL-2 receptor common gamma chain (the IL-2Rγ_c) receptor subunit *via* STAT5 (35). SOCS1 most commonly interacts directly with JAK1, JAK2, and TYK2 including in their dephosphorylated states, with its major mechanism of action being to inhibit the activity of these JAKs through the pseudosubstrate KIR, with the SOCS box of SOCS1 having a poor affinity for E3 ligase components (57). SOCS1 can also bind to phosphotyrosine residues on cytokine receptor chains such as IL-2Rβ through its SH2 domain (39). Through these mechanisms SOCS1 regulates a raft of cytokines central to the control of immunity and inflammation, including IFN-γ and downstream STAT1 (32) and various IL-2R γ_c subunit utilizing cytokines and downstream STAT5 and STAT6 (38, 79).

Transgenic expression of SOCS1 in T cells resulted in an increased CD4+/CD8+ T cell ratio and peripheral T cell defects, with reduced IFN-γ-induced STAT1 activation (80). In contrast, SOCS1$^{-/-}$ mice succumbed to early lethality at 2–3 weeks post-gestation due to pathological inflammation characterized by T cell activation (81, 82), which could be substantially alleviated through injection of anti-IFN-γ or by crossing onto a IFN-γ deficient background, suggesting that excessive IFN-γ signaling was largely responsible (32). Transgenic expression of SOCS1 with a deleted SOCS box in the SOCS1$^{-/-}$ mice also facilitated enhanced survival, highlighting the importance of KIR-mediated negative regulation (83). Moreover, the inflammatory phenotype could be mitigated by crossing onto a RAG2 deficient background (81) or in thymocyte-specific SOCS1 knockout mice (84), indicating lymphocytes were responsible for much of the inflammation. However, SOCS1$^{-/-}$ IFN-γ$^{-/-}$ mice still displayed markedly lower numbers of total T cells, reduced CD4/CD8 T cell ratio, and increased T cell activation (85). The

TABLE 1 | Mechanisms of SOCS action on cytokine signaling in immune cells.

SOCS protein	Mechanism of action	Cytokine	STAT	References
SOCS1	JAK inhibition	IFN-γ	STAT1	(32)
		IFN-α/β	STAT1/2	(33)
		IL-12	STAT4	(34)
		IL-2,7,15,21	STAT5	(35–37)
		IL-4	STAT6	(38)
	Competitive binding	IL-2,15	STAT5	(39)
SOCS2	JAK inhibition	IL-2,15	STAT5	(40, 41)
		IL-3, GM-CSF	STAT5	(42)
		IL-4	STAT6	(40)
SOCS3	JAK inhibition	IL-6, 23, G-CSF	STAT3	(43–45)
	Degradation/re-routing	IL-12	STAT4	(46)
		G-CSF	STAT3	(47)
CISH	Competitive binding	IL-12	STAT5	(48)
	Degradation/re-routing	IL-15	STAT5	(49)
		IL-3, GM-CSF	STAT5	(50)
	?	IL-4,13	STAT6	(51)
SOCS5	Competitive binding	IL-4	STAT6	(52)
SOCS4,6,7	?	?	?	

?, not known.

T cells in these mice were hypersensitive to a range of cytokines that utilize IL-2Rγ_c, including IL-2 and IL-4 and IL-7, resulting in enhanced proliferation and survival (84, 85).

SOCS1 influences the development of CD8+ T cells, with both SOCS1$^{-/-}$ and SOCS1$^{-/-}$IFN-γ$^{-/-}$ mice exhibiting a significant increase in CD8+ T cells (86, 87). This was mediated by enhanced signaling by the cytokines IL-7, IL-15, and IL-21 which stimulate CD8+ T cell production (88, 89). In the absence of SOCS1, STAT5 was constitutively activated by IL-7 and IL-15, stimulating pathological generation of CD8+ T cells (79, 86), which was blunted in both SOCS1$^{-/-}$IL-7$^{-/-}$ and SOCS1$^{-/-}$IL-15$^{-/-}$ mice (36). The excessive IL-15 signaling also impaired elimination of auto-reactive CD8+ T cells thereby contributing to autoimmunity (79).

SOCS1 has additionally been shown to control the polarization of CD4+ T cells toward their various subtypes (35) (**Figure 4**). Induction of SOCS1 by IL-4 and IL-6, as well as by IFN-γ itself, directly inhibits IFN-γ-mediated STAT1 activation that facilitates Th1 differentiation (34, 90). Accordingly, deficiency of SOCS1 in CD4+ T cells resulted in an elevated Th1 response as a direct result of enhanced IFN-γ signaling, but also indirectly through reduced IL-6/STAT3-mediated Th1 suppression mediated by enhanced levels of SOCS3 (87). As a corollary, overexpression of SOCS1 in CD4+ T cells suppressed Th1 differentiation (90). SOCS1 can also inhibit both IL-4-mediated STAT6 activation that drives Th2 polarization, and IL-12-mediated STAT4 that drives Th1 polarization, with loss of SOCS1 resulting in enhanced signaling *via* both pathways and increased Th cell differentiation (91). SOCS1$^{-/-}$ IFN-γ$^{-/-}$ knockout mice were skewed toward a Th2 response, highlighting the role of SOCS1 in the inhibition of Th2 polarization (92). Loss of SOCS1 in CD4+ T cells additionally suppressed Th17 cell development, with enhanced IFN-γ signaling antagonizing transforming growth factor (TGF)-β mediated SMAD activation and also indirectly blunting IL-6 mediated STAT3 signaling (87). SOCS1 has been further demonstrated to suppress STAT5 activation in response to IL-2, providing an alternative mechanism to inhibit differentiation of Th17 cells (93, 94). SOCS1 is also required for the maintenance and activity of Foxp3+ regulatory T (Treg) cells (95). Interestingly several similarities exist between SOCS1$^{-/-}$ mice and the Treg-deficient *scurfy* mice, both of which face premature death 2–3 weeks after birth and display inflammatory phenotypes associated with increased IFN-γ signals (81, 96). SOCS1 is highly expressed in Treg cells, but T cell-specific over expression of SOCS1 was associated with decreased Treg cells whereas T cell-specific knockout resulted in increased Tregs in the thymus (97). This was due at least partially to the ability of SOCS1 to inhibit IL-2-mediated STAT5 activation that contributes to the proliferation of these cells (97, 98). SOCS1$^{-/-}$ mice were however deficient in peripheral Treg cells despite the enhanced thymocyte development, with adoptive transfer of SOCS1$^{+/+}$ Treg cells able to reduce inflammation and increase survival (99). Treg-specific SOCS1 ablation resulted in a loss of suppressor functions (98). SOCS1$^{-/-}$ Treg cells also showed hyperactivation of STAT1 and STAT3, enhancing the production of IFN-γ and IL-17, respectively, with Foxp3 expression rapidly lost, and these cells converting to Th1- or Th17-like cells (95). This decrease in Foxp3 expression was rescued by deletion of IFN-γ, showing that SOCS1 inhibits IFN-γ mediated STAT1 activation that would otherwise suppress Foxp3 expression (95). In addition, SOCS1 is required in Treg cells to suppress the IL-12-mediated activation of STAT4, which modifies the Treg cells into an inflammatory Th1-like cell (34).

SOCS1$^{-/-}$ mice showed altered natural killer T (NKT) cells, with a decrease in invariant NKT cells but an increased activation

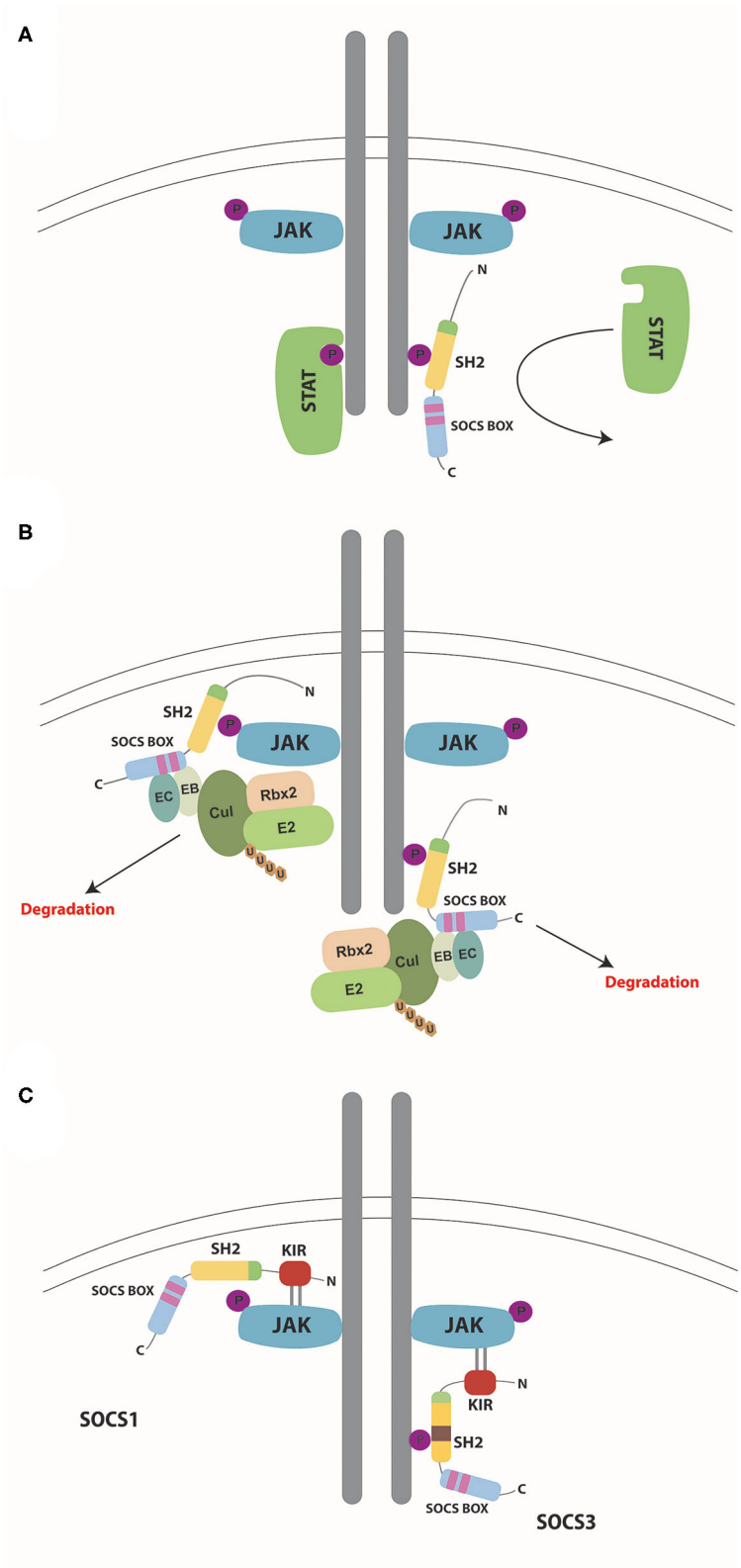

FIGURE 3 | Mechanisms of SOCS action. Schematic representation of the mechanisms by which SOCS proteins negatively regulate cytokine receptor signaling via the JAK/STAT pathway. **(A)** Competitive binding. SOCS proteins bind to phosphorylated tyrosine residues on the cytokine receptor via their SH2 domain, thereby preventing docking of STAT (and other) proteins and so inhibiting their subsequent activation. **(B)** Degradation. SOCS proteins bind through their SH2 domains to

(Continued)

FIGURE 3 | phosphorylated target proteins such as receptor-associated JAK proteins (left) or receptors themselves (right), subsequently assembling components of the E3 ubiquitin ligase complex: elongins B and C binding to the BC box and Cullin 5 binding to the Cul box located within the SOCS box. Other components of the complex such as Rbx2 and E2 are assembled through interactions with Cullin 5. Ubiquitin molecules are transferred from the ligase to the target protein substrates, which signals their degradation along with that of associated proteins within the complex. **(C)** Inhibition of kinase activity. Both SOCS1 and SOCS3 utilize their SH2 domain to bind to phosphorylated tyrosine residues on either JAKs (left) or receptors (right), respectively. Both then directly inhibit JAK kinase activity via their KIR domain.

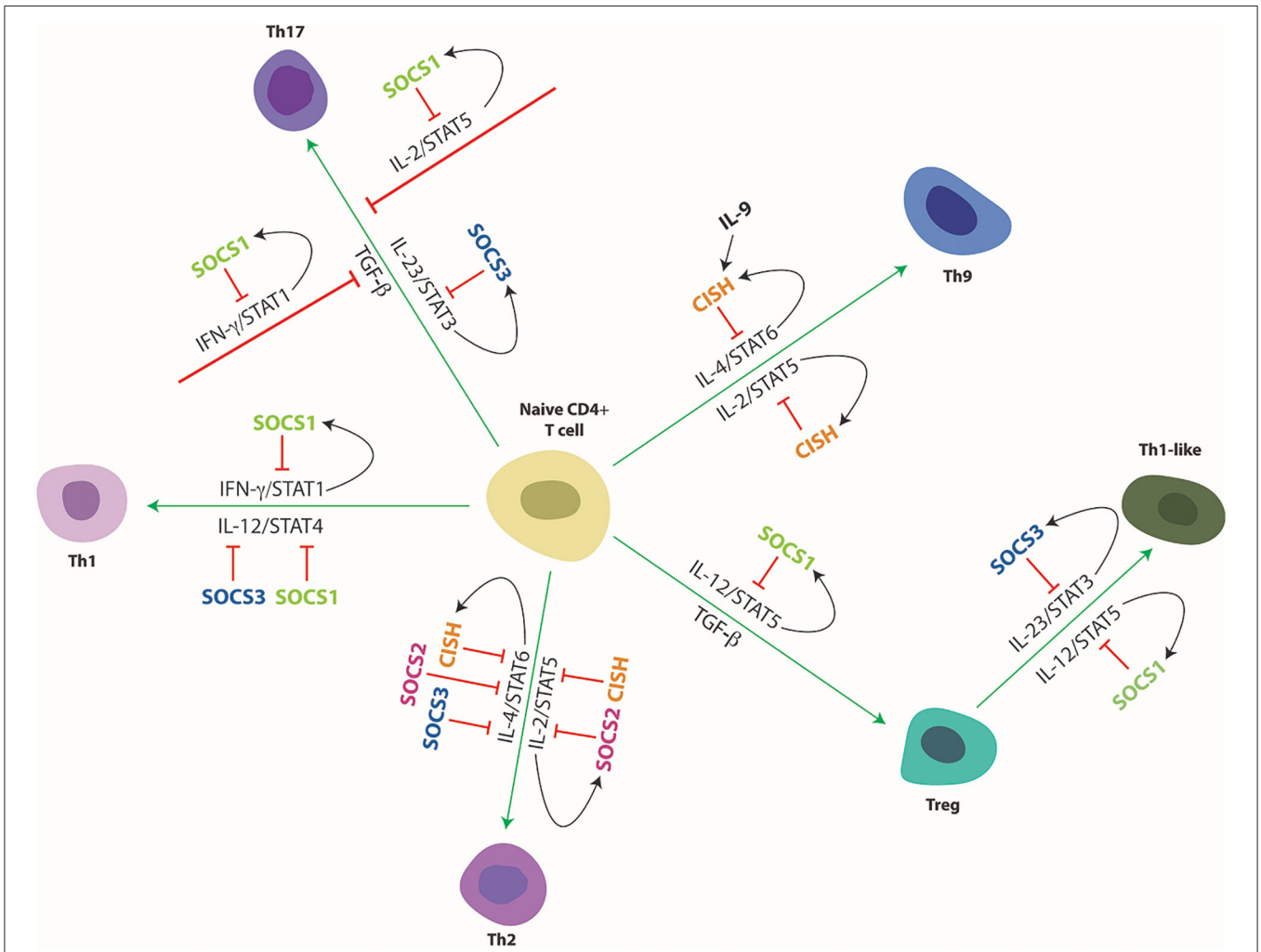

FIGURE 4 | Cytokine mediated differentiation of naïve CD4+ T cells to their various subsets and its regulation by SOCS proteins. Schematic representation of naïve CD4+ T cell differentiation toward the indicated subtypes and its control by specific cytokines and downstream STAT proteins and their regulation by specific SOCS proteins. Green arrows indicate stimulation and red lines indicate suppression of developmental pathways, with black arrows delineating SOCS induction.

of conventional NKT cells that supplemented the inflammation and autoimmunity observed in these mice (84). This was due to enhanced IFN-γ activation and IL-2 and IL-15 proliferative signaling (100). NKT cells displayed sustained IFN-γ and IL-4 signaling in the absence of SOCS1, including loss of the cross-inhibitory action of IFN-γ on IL-4. This resulted in enhanced NKT cell activation that stimulated inflammation, which could be alleviated either by removing NKT cells or by deletion of STAT1 or STAT6 which are activated by IFN-γ and IL-4, respectively (38).

SOCS1 also influences the differentiation and function of dendritic cells (DCs) to suppress systemic autoimmunity and maintain their tolerogenic phenotype. Restoration of SOCS1 expression in T and B cells of SOCS1$^{-/-}$ mice resulted in accumulation of DCs in the thymus and spleen, and pertubed activation of these cells due to hyperresponsiveness to IFNγ and IL-4, which resulted in aberrant B cell expansion and autoreactive antibody production (101). Autoimmunity in SOCS1$^{-/-}$ mice was largely mediated by a specific increase in CD8+ DCs, resulting in increased IFNγ and IL-12 production

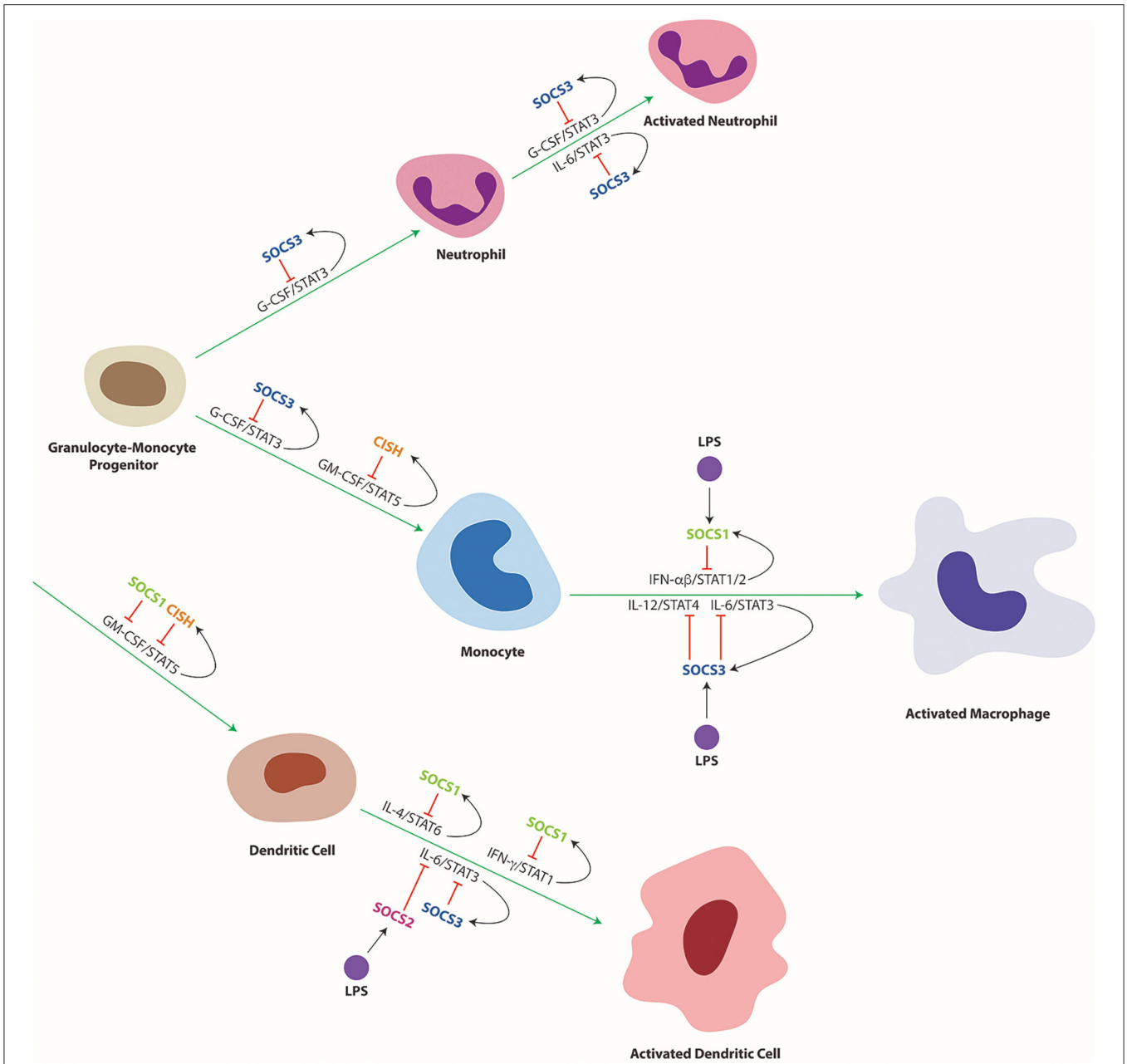

FIGURE 5 | Cytokine mediated differentiation and activation of myeloid cells and its regulation by SOCS proteins. Schematic representation of myeloid cell differentiation along the indicated lineages, including their subsequent activation as well as its control by specific cytokines and downstream STAT proteins and their regulation by specific SOCS proteins. Green arrows indicate stimulation and red lines indicate suppression of developmental pathways, with black arrows delineating SOCS induction.

(102). DCs from SOCS1$^{-/-}$ mice exhibited stronger Th1 responses, also including increased IFNγ production by T cells (103). The mechanisms by which SOCS1 regulates DC development remain controversial, but SOCS1 expression can block GM-CSF-mediated signaling and differentiation of DCs, with SOCS1 shown to induce the ubiquitin-mediated degradation of the GM-CSF-R βc subunit (104) (**Figure 5**).

SOCS1$^{-/-}$ mice additionally display increased numbers of eosinophils and macrophages, although this appears to be largely an indirect effect of aberrant T cells in these mice rather than impacts on their development *per se*. SOCS1 is rapidly induced by Toll-like receptors (TLR) in response to ligands such as lipopolysaccharide (LPS) and has been found to influence the activation and subsequent polarization of macrophages (105). Indeed, SOCS1$^{-/-}$ mice challenged with LPS show

augmented innate immune responses characterized by enhanced macrophage activation, including high levels of tumor necrosis factor (TNF)-α and IL-12 (106, 107). However, the mechanism of action remains somewhat controversial. Several studies have indicated that the effect is indirect, with the SOCS1 induced by TLRs blocking downstream IFNα/β signaling (33, 108). In contrast, others have shown that SOCS1 can directly negatively regulate TLR signaling through interactions with various adapter proteins that such as IL-1 receptor associated kinase (106) and Mal/TIRAP (109) which are responsible for the induction of inflammatory genes *via* NF-κB. Intriguingly, even in the absence of IFN-γ and STAT1, SOCS1$^{-/-}$ mice were hypersensitive to LPS, showing that this regulation is at least partially distinct from the effect of SOCS1 on IFN signaling (106). Either way SOCS1 plays significant immunosuppressive roles in the myeloid lineage mitigating the development of excessive inflammation (110).

With the strong role of SOCS1 in the regulation of multiple aspects of immunity, it is perhaps not surprising that SOCS1 impacts on a variety of inflammatory diseases. T- and NK-cell specific SOCS1 knockout mice displayed increased sensitivity to ConA-induced hepatitis, due hyperresponsive of these cells to IL-2 and IL-15 (100). SOCS1$^{+/-}$ mice and those with T cell-specific SOCS1 ablation were more susceptible to experimentally-induced colitis due to enhanced IFNγ/STAT1-mediated suppression of Treg cells (111). SOCS1$^{-/-}$ mice also showed enhanced formation of atherosclerotic plaques due to increased M1 macrophages and neutrophils (112). In contrast, T cell-specific SOCS1 knockout mice were resistant to experimental autoimmune encephalomyelitis (EAE) in this case a result of enhanced IFNγ/STAT1-mediated suppression of Th17 cells that drive this model of autoimmunity (87). Moreover, transgenic SOCS1 expression in T cells resulted in the development of intestinal inflammation (113). Mice with DC-specific SOCS1 ablation, showed a skewed immune response to bacterial antigen, with reduced CD8+ T cells and NK cells but enhanced innate responses (114). Knockdown of SOCS1 in DCs resulted in reduced susceptibility to *Candida albicans*, with increased phagocytosis and killing by DCs (115), but also increased Th1 cells and elevated serum IFN-γ that blunted inflammation during late stages of infection (116). Altered SOCS1 expression has been observed in patients with systemic lupus erythematosus (117) and rheumatoid arthritis (118), with SOCS1 polymorphisms demonstrated to predict severity of the latter disease (119). Recently, heterozygous loss-of-function germline mutations in SOCS1 have been shown to be associated with early onset human autoimmune diseases, with lymphocytes showing hyperactivation concomitant with increased STAT activation in response to IFN-γ, IL-2, and IL-4 that is reverted with a JAK1/2 inhibitor (120).

SOCS1 has additionally been implicated in a variety of immune cell malignancies, with silencing of SOCS1 due to methylation or mutation commonly reported, reflective of the negative regulatory roles played by SOCS1 in the cytokine-mediated proliferation, differentiation and survival of immune cells (121, 122). For example, SOCS1 is frequently methylated in cases of acute (AML) and chronic myeloid leukemia (CML) (123–125), which is thought to block the ability of SOCS1 to negatively regulate JAK2 activity through kinase inhibition (126), thereby promoting activation of STAT3 and STAT5 which are major drivers of leukemia development (123). However, SOCS1 deficiency can also impact on therapeutic responses, being associated with poor outcomes to IFN-α treatment in AML (127). In CML, SOCS1 is strongly induced by BCR-ABL, but its role is complex, since it can inhibit both pro-proliferative responses mediated by IL-3 and IL-6, as well as anti-proliferative responses to IFNs. In a mouse model, BCR-ABL-dependent growth was resistant to SOCS1 expression, but a subset of SOCS1-expressing mice showed extended disease latency or indeed an absence of disease (128). SOCS1 methylation has also been observed in multiple myeloma, where it correlated with enhanced STAT3 activation and was associated with poorer prognosis (129). Loss-of-function somatic mutations of SOCS1 have also been frequently observed in a range of B cell malignancies. These include Hodgkin's lymphoma (130–132), where they are associated with shorter patient survival (133), SOCS1 deficiency in this disease correlated with hyperactivation of JAK2 and downstream STAT6 leading to excessive proliferation (134), with SOCS1 mutations synergizing with presumed gain-of-function STAT6 mutations (135). Somatic SOCS1 mutations have also been commonly found in diffuse large B cell lymphoma (DLCBL) (131, 136), where they contribute to unrestrained IL-6 signaling (137). Interestingly, patients with such mutations responded less well to anti-CD20 therapy resulting in reduced survival (138). Similar loss-of-function mutations have been reported in gray zone lymphoma (139), primary mediastinal B cell lymphoma (PMBCL) (140) and HIV-associated plasmablastic lymphoma (141). SOCS1 has also been found to be mutated in malignancies involving other lymphoid malignancies, including T cell prolymphocytic leukemia (T-PLL) (142), NK/T cell lymphoma (143), enteropathy-associated T cell lymphoma (EATL) (144), as well as mycosis fungoides, a cutaneous T cell lymphoma (CTCL) (145). Indeed, in CTCL cells, SOCS1 knockdown was shown to increase aggressiveness, with cooperation seen with activating JAK3 mutations (93).

SOCS2

SOCS2 is strongly activated by and in turn negatively regulates STAT5 and to a lesser extent STAT6 and STAT3 (146, 147). The negative regulation is principally mediated by SOCS box-dependent degradation of target substrates (148), including receptors and downstream signaling molecules (149). Intriguingly SOCS2 also regulates other SOCS proteins, particularly SOCS1 and SOCS3 (150, 151), but in a dose-dependent manner (152). Through this mechanism SOCS2 can actually indirectly stimulate cytokine-induced STAT activation by removing the negative regulation mediated by those SOCS proteins targeted (151). SOCS2 is primarily involved in regulation of developmental and homeostatic pathways, such as those mediated by growth hormone, insulin-like growth factor and prolactin signaling (153, 154). However, more recent studies have illuminated novel functions of SOCS2 within the immune system.

SOCS2$^{-/-}$ mice showed no obvious basal defects in T cells (40, 155–157). However, SOCS2 has been demonstrated to

influence lymphoid cell activation and polarization (**Figure 4**). In the absence of SOCS2, TCR stimulation of CD4+ T cells resulted in enhanced levels of IL-4, IL-5 and IL-13 thereby stimulating the differentiation of Th2 cells at the expense of Th17 cells (40). SOCS2 was also found to be induced by IL-4 and IL-6, with loss of SOCS2 leading to enhanced activation of STAT5 and STAT6 in response to IL-2 and IL-4, respectively, but blunted IL-6-mediated STAT3 activation potentially mediated indirectly by SOCS1 and SOCS3 which were upregulated in SOCS2$^{-/-}$ mice (40). Thymic Foxp3^{+} Treg cells were unaffected in SOCS2$^{-/-}$ mice, but enhanced IL-4-induced STAT6 peripheral inducible Treg cells (iTreg) were observed, resulting in increased levels of inflammatory cytokines such as IL-13 and IFN-γ (156). This suggests that SOCS2 is required to conserve the intrinsic anti-inflammatory phenotype of iTreg cells (156).

NK cells were elevated in the bone marrow and spleen of SOCS2$^{-/-}$ mice (41). This was shown to be a cell autonomous effect, with SOCS2 able to inhibit IL-15 signaling specifically through the JAK2/STAT5 pathway, but not the JAK1/STAT3 pathway, mediated *via* direct interaction with JAK2. This caused increased NK cell differentiation and although these cells showed unaltered cytolytic activity and IFNγ secretion, SOCS2$^{-/-}$ mice showed enhanced resistance to melanoma (41). SOCS2 was also found to be induced by IL-15 in human NK cells, although in this case it was found to target phosphorylated proline-rich tyrosine kinase 2 for proteasomal degradation, leading to enhanced cytolytic activity and other effector functions, including secretion of inflammatory cytokines, in these cells (158).

SOCS2 deficiency also impacted on hematopoietic stem cells (HSCs) (42). In these cells, SOCS2 was induced by IL-3, GM-CSF, and thrombopoietin (TPO) *via* STAT5 activation in HSCs, with proliferation mediated by these cytokines increased in HSC derived from SOCS2$^{-/-}$ mice. There was also unrestrained myelopoiesis following bone marrow ablation in these mice, which leads to exhaustion of long-term HSC (42).

Expression of SOCS2 has been shown to be markedly increased during maturation of DCs, both mouse (159) and human (160). Even though its role in DC production is unclear, SOCS2 is induced by ligands such as LPS and Lipoxin A_4 that signal through TLR4 and TLR2, respectively (161, 162), although this is probably caused by indirect induction of SOCS2 stimulated by type 1 IFNs produced in DCs in response to LPS in a unique autocrine-paracrine loop (161). Indeed, SOCS2 is required for the propagation of MyD88-dependent and -independent signaling pathways downstream of TLR4 (160). As a result, in the absence of SOCS2, LPS induced expression of inflammatory cytokines such as TNFα, IL1-β, and IL-6 was markedly diminished (160). However, another study showed SOCS2 suppression in DCs caused hyperphosphorylation of STAT3 resulting in an increase in inflammatory cytokines (163). In contrast, SOCS2 did not influence TLR signaling in mouse macrophages (33).

SOCS2 has been shown to suppress inflammation in a range of disease models, but the cellular mechanism differs substantially depending on the model. Thus, in models of atopic dermatitis and allergen-induced airway inflammation SOCS2$^{-/-}$ mice showed enhanced allergic responses with increased IgE, eosinophilia and inflammatory pathology attributable to increased Th2 responses, which were also seen following helminth challenge (40). However, in EAE, SOCS2 deficiency resulted in reduced acute phase damage due to increased Th17 and decreased Th1 and Th2 cells, but exacerbated inflammation during the late phase of disease, characterized by increased Th1 cells and decreased Th2 and Treg cells (164). In a mouse model of cerebral malaria SOCS2$^{-/-}$ mice initially showed reduced parasitemia, however at late stages they showed increased parasitemia associated with increased Th1 and Th17 cells and pro-inflammatory cytokines such as IL-6 and IL-17 with decreased Treg cell activation (165). Interestingly, the aberration in T cell ratios and abnormal cytokine production were alleviated when mice were treated with a nitric oxide synthase (NOS) inhibitor, suggesting a role for SOCS2 in regulating NO synthesis (165). In comparison, SOCS2$^{-/-}$ mice showed reduced parasitemia and decreased IFNγ and TNFα following *T. cruzi* infection, which was associated with increased generation of Treg cells but reduced memory T (Tm) cells (166).

SOCS2 has also been implicated in various malignancies (167, 168), notably including myeloproliferative disorders and leukemias although this can be both pro- and anti- tumorigenic. As a major STAT5 target gene, its expression is strongly increased in a range of myeloproliferative disorders where STAT5 is activated, including BCR/ABL-induced CML (169) and JAK2 V617F-induced disease (170). However, SOCS2 has been shown to be dispensable for the induction and propagation of BCR/ABL-mediated disease (155). In contrast, it was demonstrated to be a negative regulator of JAK2 V617F and was epigenetically downregulated in MPD patients (170). Moreover, SOCS2 expression predicted poor outcomes in pediatric AML (171), while high levels of SOCS2 correlated with progression for both myeloid and lymphoid leukemias (42). In this case SOCS2 expression was under the control of the myocyte-specific enhancer factor 2C and was involved in maintaining the stemness of the leukemias (42). SOCS2 has also been identified as a prognostic signature for the development and progression of AML (172). SOCS2 knockdown reduced growth of AML cells *in vitro* and delayed disease upon transplantation into mouse models, with these cells showing mature myeloid cell markers (172), suggesting a potential oncogenic role for SOCS2.

SOCS3

SOCS3 is principally induced by cytokines that activate STAT3 *via* JAK2, such as those that utilize the GP130 subunit [for example, IL-6, IL-10, and leukemia inhibitory factor (LIF)], or related receptor chains (G-CSF and leptin) but also by those that activate STAT1 (such as IFN-γ), and STAT5 [such as erythropoietin (EPO)] (173). The major mechanism of action for SOCS3 is through docking to cytokine receptors *via* its SH2 domain allowing the KIR to inhibit the activity of adjacent JAKs, but it can also compete with STAT docking and mediate proteasomal degradation (174, 175). It plays a particularly important role in immunity and inflammation *via* its negative regulation of members of the IL-6 family of cytokines that act *via* STAT3, notably including IL-6 and G-CSF (58).

SOCS3$^{-/-}$ mice display embryonic lethality (176, 177), principally as a result of placental defects caused by excessive

LIF signaling, with no direct impact on early hematopoiesis (177, 178). Hematopoietic-specific SOCS3 ablation was able to overcome these effects but resulted in the development of a lethal inflammatory disease, characterized by splenomegaly, pericarditis, hepatitis, and neutrophilia (43). This was largely a macrophage and neutrophil-driven process, although non-hematopoietic tissues have also been implicated (179). Hematopoietic-specific SOCS3 knockout mice display elevated IL-6 (43), and are highly susceptible to IL-1β-mediated inflammation leading to rapid mortality (180). IL-6 deficiency ameliorated the inflammatory disease, including that mediated by IL-1β (179). SOCS3 ablation prolonged STAT3 activation by IL-6 (44, 181), but interestingly also resulted in extended STAT1 activation by IL-6, which promoted a IFN-like response, but IL-10 responses were not surprisingly impacted (181). SOCS3 has been shown to dock directly to the GP130 subunit of the IL-6R to suppress signaling (182), with mice harboring a specific mutation at this site (Y759F) displaying similar splenomegaly, lymphadenopathy, B and T cell defects and autoimmune arthritis to SOCS3-deficient animals (183), highlighting the key role of perturbed IL-6R signaling.

SOCS3 knockout did not affect the overall numbers of CD4+ or CD8+ T cells (45, 184), but SOCS3 is believed to influence the polarization of T helper cells (**Figure 4**). SOCS3 is able to directly block IL-12-mediated STAT4 activation through competitive binding to STAT4 docking sites on the IL-12Rβ2 subunit (46), with high levels of SOCS3 correlating with reduced activation of STAT4 by IL-12 that induces the production of IFN-γ thereby inhibiting Th1 differentiation and favoring Th2 differentiation (184, 185). Enforced expression of SOCS3 impacted T cell development in the thymus and peripheral T cell homeostasis through impaired IL-7-mediated signaling (186), and also inhibited Th cell proliferation (187), presumably through blocking IL-2 production (188, 189). Despite this, T cell specific deletion of SOCS3 was found to suppress production of both Th1 and Th2 cells, as a result of enhanced IL-10 and TGF-β mediated STAT3 activation, producing an anti-inflammatory response resulting in an increase in regulatory Th3 and Th17 cells (190). The increased Th17 cells appears to be due to the loss of negative regulation of IL-23 signaling by either a feedback loop (45) or by crosstalk from inflammatory cytokines such as IFN-γ and LIF (191, 192), as well as ablation of TGF-β-mediated inhibition of IL-6 signaling (193), which each serve to increase STAT3 activation and thus drive Th17 differentiation. Increased expression of pro-inflammatory genes by macrophages from SOCS3-deficient mice further supports Th1 and Th17 differentiation (194). Deletion of SOCS3 in CD4+ T cells revealed an essential role in maintaining the expression of costimulatory molecules CD28 and CTLA-4 and for the induction of IL-2 upon TCR stimulation, that resulted in expansion of Treg cells at the expense of Th17 and Th1 (184). This is consistent with impaired Treg production and immune suppressive capabilities leading to defective self-tolerance following overexpression of SOCS3 in Foxp3+ Treg cells (195) as well as in SOCS3 transgenic mice (186). Indeed, SOCS3 was also shown to serve as a negative regulator of Foxp3 expression to directly inhibit Treg function (196). SOCS3 is also expressed in NKT cells, in response to cytotoxic cytokines such as IL-10 (197), playing an immunosuppressive role through regulation of cytokine signaling (198). Finally, SOCS3 expression in thymic stromal cells has also been demonstrated to be important for normal T cell development (199).

SOCS3 also plays a role in DCs to regulate their tolerogenic capabilities. IL-6 was shown to induce the expression of SOCS3 in DCs, with SOCS3-deficient DCs exhibiting enhanced STAT3 activation and increased tolerogenic activity compared to their wildtype counterparts (200). In human DCs, SOCS3 was shown to induce proteasomal degradation of the key enzyme indoleamine 2,3-dioxygenase (201), providing an additional mechanism by which it can suppress tolerogenic activities.

SOCS3 has several important functions within the myeloid lineage, controlling the differentiation and activation of both neutrophils and macrophages (**Figure 5**) (202). In neutrophils, G-CSF induces SOCS3 *via* STAT3 activation (203), facilitating negative feedback regulation to maintain G-CSF signaling at homeostatic levels (204). SOCS3 binds directly to the activated G-CSF receptor (G-CSFR) and inhibits signaling through either suppression of JAK2 activity (58) or by ubiquitination of the G-CSFR thereby stimulating the rerouting of the receptor through lysosomes (47). As a consequence, $SOCS3^{-/-}$ mice exhibit excessive G-CSF signaling resulting in neutrophilia (178). HSC-specific SOCS3 ablation resulted in increased colony size in response to G-CSF, whereas neutrophil-specific ablation resulted in neutrophilia, with enhanced and extended G-CSF signaling and increased survival (205). Disruption of SOCS3 in hematopoietic cells also triggered the development of neutrophilia and inflammatory pathologies in later adulthood, including infiltration of neutrophils into multiple tissues such as liver, lung, and muscle tissue accompanied by elevated IL-6 and G-CSF levels. Stimulation of these mice by G-CSF triggered increased progenitor proliferation and enhanced neutrophilia and increased formation of both macrophage and neutrophil colony formation (43). This ultimately resulted in cerebral infiltration by neutrophils hyperactivated by G-CSF (206). Surprisingly, G-CSF increased the formation of both neutrophil and macrophage colonies (43), with the differentiation of progenitors skewed toward macrophages in response to G-CSF or IL-6 (207).

Within the macrophage lineage there is evidence that SOCS3 has both pro-inflammatory as well as anti-inflammatory roles. SOCS3 suppresses the inflammatory activities of macrophages by providing negative feedback regulation of inflammatory cytokines such as IL-6, IL-1β, and IL-12, with myeloid-specific SOCS3 knockouts showing enhanced and prolonged JAK/STAT signaling, increasing the levels of M1 pro-inflammatory genes (194). Furthermore, LPS and TNF-α are also able to induce SOCS3 to provide additional negative regulation of IL-6-mediated STAT3 activation (208, 209), with bacterial infections associated with enhanced SOCS3 expression in macrophages, presumably as an alternative mechanism to suppress their inflammatory capabilities (210, 211). Hence, it can be predicted that SOCS3 attenuates STAT3 activation thereby promoting macrophage activation *via* positive regulation by TGF-β and IL-6 (212). Independent of this pathway, knockdown of SOCS3 in

myeloid cells resulted in an increase in phosphatidyl insotiol-3-kinase (PI3K)-AKT signaling which is known to suppress the production of IL-6 and induction of NF-κB, providing an alternative mechanism for SOCS3 to promote inflammation (212, 213). However, LPS-induced SOCS3 in macrophages also indirectly stimulated the induction of inflammatory genes such as NF-κB, although the mechanism that has not been identified (213, 214). These opposing effects could be attributed to the kinetics of STAT3 signaling, and the antagonistic roles of IL-6 on LPS signaling (211). Lastly, in the absence of SOCS3 the phagocytic abilities of macrophages were found to be heightened, attributed to enhanced IL-6 and IL-12 signaling (215, 216). Intriguingly, PI3K-AKT was found to contribute to the heightened ability of macrophages to undergo phagocytosis by promoting the cytoskeletal rearrangement required for this process, with inhibition of PI3K signaling in SOCS3 silenced cells found to suppress the heighted phagocytic capabilities (217).

A variety of disease models have highlighted critical roles for SOCS3 in both the development and resolution phases of inflammation (218). In mouse models of inflammatory arthritis, SOCS3 deficiency in the hematopoietic compartment caused more severe joint inflammation associated with elevated levels of IL-6 and G-CSF, increased neutrophil numbers and constitutively activated CD4+ T cells and macrophages (180). Mice with myeloid-specific SOCS3 ablation also develop more severe EAE (216, 219) and show enhanced LPS induced sepsis (194) and acute lung injury (220). In addition, they are susceptible to experimentally-induced aortic dissection associated with enhanced STAT3 activation in macrophages and increased inflammation (215). In contrast, CD4+ T cell-specific SOCS3 ablation provided protection from uveitis due to an expansion of Treg cells at the expense of Th17 cells (184). Moreover, acute nephritis was associated with increased SOCS3 activation that facilitated activation of macrophages toward a pro-inflammatory M1 phenotype with increased iNOS production (213). SOCS3 deficiency in either the myeloid or lymphoid lineage independently increased susceptibility to *M. tuberculosis* (202). Ablation of SOCS3 within myeloid cells resulted in enhanced IL-6 signaling which inhibited the secretion of TNF-α and IL-12 required to mediate the development of a robust CD4+ T cell response against the infection (221), as well as increased production of NOS2 and Arg1, leading to higher bacterial loads and exacerbated pulmonary inflammation (210). DC-specific SOCS3 ablation resulted in increased susceptibility to infection as a result of reduced cross-talk with T cells (218). Separately, SOCS3 deficiency in lymphoid cells resulted in an increase in IL-17 secretion during *M. tuberculosis* infection, which mediated enhanced neutrophilic inflammation (221). Hematopoietic-specific SOCS3 deficiency also caused increased sensitivity to lymphocytic choriomeningitis virus-mediated lethality (179). Finally, in mice models of inflammatory arthritis, SOCS3 induction alleviated bone degradation and significantly suppressed the autoimmune inflammation within the joints (222).

As a corollary, SOCS3 has also been implicated in specific human inflammatory diseases. These include inflammatory bowel disease (IBD) and its more severe phenotype, Crohns disease (223, 224), with SOCS3 responsible for suppressing the activation of inflammatory genes induced by STAT3 during IBD (224). In mice, activation of STAT3 and expression of SOCS3 is characteristic for the development of IBD and colitis, with SOCS3 acting as a regulatory mechanism to limit pathogenesis, which is primarily driven through macrophages (225). In addition, inflamed tissue obtained from IBD patients showed an increase in classically activated inflammatory macrophages, with a large majority of these cells displaying an upregulation of SOCS3, a reflection of the roles played by SOCS3 in macrophage polarization (212, 213). Indeed, increased SOCS3 expression predicts mucosal relapse in ulcerative colitis (226). Similarly, SOCS3 expression in human arthritic chondrocytes contributed to cartilage damage during arthritis (227, 228).

Due to its role in controlling the activation of the STAT3 oncogene, SOCS3 is typically considered a tumor suppressor protein, although this is not always the case in immune cancers (229). Loss of SOCS3 expression, particularly *via* promoter methylation, has been identified in a variety of hematopoietic malignancies. Thus, SOCS3 methylation occured at high frequency in BCR-ABL-negative chronic myeloproliferative disease (CMPD) and post-CMPD acute myeloid leukemia (230), as well as chronic lymphoproliferative disease of NK cells (CLPD-NK) (231). SOCS3 hypermethylation in multiple myeloma was associated with drastically shortened patient survival, believed to be due to enhanced responsiveness to IL-6, a major driver of this disease (232). SOCS3 expression was also ablated in Lck LSTRA leukemia, with enforced ectopic expression of SOCS3 reducing cell proliferation and increasing apoptosis in Lck-transformed cells (233). Decreased SOCS3 expression due to methylation was detected in a considerable proportion of mantle cell lymphoma (MCL) patients, with a trend for worse outcomes in this cohort. Enforced re-expression of SOCS3 reduced IL-10-mediated STAT3 activation and increased apoptosis (234–236). SOCS3 hypermethylation was also common in idiopathic myelofibrosis, but not other MPDs, although no significant correlation with survival or other clinical parameters was found, whereas SOCS3 expression was increased in JAK2 V617F-positive myeloproliferative disorders (MPDs) (234–236). SOCS3 was consistently down-regulated in CML cell lines and bone marrow nuclear cells (BMNCs) from CML patients, with enforced expression inhibiting growth (237). Moreover, in cases where SOCS3 was expressed, it enhanced cell survival as a result of BCR-ABL-mediated phosphorylation (238). However, constitutive SOCS3 expression in CML conferred resistance to IFNα (239, 240). Similarly, in CTCL constitutive SOCS3 expression was found to play a protective role by blocking IFN-α mediated growth suppression and differentiation without impacting STAT3 activation (241). Indeed overexpression of SOCS3 in the peripheral blood of non-Hodgkin lymphoma (NHL) patients correlated with advanced disease and a poor response to treatment (242). In one form of this disease, *de novo* follicular lymphoma with $t_{(14;18)}$, SOCS3 expression induced by overexpressed BCL2 was associated with poor prognosis (243, 244). Ablation of SOCS3 within the myeloid lineage was found to promote tumor development in mice. This was mediated by tumor-derived G-CSF driven proliferation of myeloid-derived

suppressor cells in the tumor microenvironment *via* STAT3, which suppressed CD8+ T cell responses against the tumor (245). In mice models of colitis associated cancer, SOCS3 deletion in intestinal epithelial cells (IEC) exacerbated the development of cancer through constitutive STAT3 activation leading to IEC proliferation (246, 247). Furthermore, downregulation of SOCS3 was also observed in patients where colitis resulted in carcinogenesis, suggesting that loss of inhibition on STAT3 promotes the development of cancer during colitis (248).

CISH

CISH is particularly relevant for cytokines that utilize STAT5, being induced largely by STAT5 and predominantly regulating STAT5 activation (19). CISH has been suggested to play a variety of roles outside the immune system, but more recent studies have identified important roles in the regulation of immune cell development and function (30, 249). Intriguingly it appears to serve as a break on both pathological inflammations, as seen in allergy and arthritis, but also anti-tumor responses (49–51).

CISH participates in T cell development mediated through its negative regulation of, principally IL-2 signaling through STAT5 and IL-4/IL-13 signaling through STAT6. This was evidenced from CISH transgenic mice, which showed increased Th2 polarization proposed to be due to suppressive effects of CISH on IL-2 signaling, including STAT5 activation (250), with CISH able to bind to the IL-2 receptor β chain component, which blocks activation of the associated JAK3 (48). A similar mechanism is likely to explain the heightened embryonic lymphopoiesis observed following knockdown of the zebrafish CISH paralogue Cish.a, which also correlated with enhanced STAT5 activation (251). CISH$^{-/-}$ mice suffered from airway inflammation and progressively developed a pulmonary disease characterized by enhanced eosinophils within the airways and epithelial cell hyperplasia (51). This was due, at least in part, to preferential differentiation of T cells into Th2 and Th9 subsets through enhanced IL-4-mediated STAT6 activation, resulting in excessive production of IL-13 and IL-9 by Th2 and Th9 cells, respectively (51) (**Figure 4**). CISH suppressed activation of STAT6 and STAT5 in response to IL-4 and IL-2 respectively, both of which drive Th9 and Th2 differentiation (51). IL-9 induced CISH expression in CD4+ T cells but CISH did not significantly effect IL-9 signaling, suggesting that IL-9 stimulates CISH in CD4+ T cells for crosstalk inhibition of other cytokines such as IL-4 (252). This contradicted previous studies where CISH overexpression resulted in elevated Th2 expression, possibly due to differences in methodology (250). Alternatively, this could also be due to compensation by other SOCS proteins in response to CISH ablation, causing an imbalance in Th1/Th2 polarization. Indeed human Th2 cells have been shown to express markedly more CISH compared to Th1 cells, supporting a negative regulatory role (253). Lastly, CISH was also able to inhibit IFN-γ signaling to some degree, although this is overshadowed by the much stronger effects of SOCS1 in most lineages, especially CD4+ T cells (254).

CISH also plays important roles in regulating the development of both CD4+ and CD8+ T cells through regulation of T-cell receptor (TCR) signaling, although there is conflicting data regarding the mechanism of action. Enforced expression of CISH in CD4+ T cells markedly increased their proliferation, survival, and activation. This resulted in enhanced MAP kinase activity in response to TCR stimulation, suggesting CISH acts as a positive regulator of TCR signaling, with CISH found to associate with protein kinase C epsilon as a potential mechanism (255). However, deletion of CISH in CD8+ T cells increased their proliferation and activity, including their cytotoxic anti-tumor capabilities (256). This suggested that CISH instead acts as a negative regulator of TCR signaling in these cells, proposed to be mediated by the ability of CISH to degrade the TCR intermediate protein PLC-γ1 rather than by impacting on STAT5 signaling (256, 257).

CISH serves a major role in controlling NK cell production and function, which is mediated by its negative regulation of IL-15. CISH is induced by IL-15, which strongly activates STAT5, and then targets IL-15R-associated JAK1 to mediate degradation of the receptor complex. In the absence of CISH the maturation and cellular turnover of NK cells in response to IL-15 is enhanced (258). However, additional mechanisms are present in order to prevent accumulation of NK cells in the absence of CISH (258). Despite this, CISH deficient NK cells showed enhanced survival and expansion even with low concentrations of IL-2 and IL-15 (259). The hypersensitivity of CISH$^{-/-}$ mice to IL-15 also enhances the anti-tumor effects of NK cells, suggesting that CISH plays a more significant role in the effector functions of NK cells rather than just their differentiation (49). Indeed, in the absence of CISH, IL-15 stimulated increased cytotoxicity of NK cells through the secretion of IFN-γ (49, 259). CISH$^{-/-}$ NK cells additionally displayed enhanced metabolic fitness attributed to increased mTOR signaling complementing their anti-tumor activities, highlighting the multiple roles played by CISH in NK cells (259).

CISH can also influence both DC and macrophage development, in this case through control of GM-CSF and IL-3 mediated STAT5 activity (**Figure 5**). CISH expression is strongly upregulated during GM-CSF-induced *ex vivo* maturation of bone marrow-derived DC which correlated with high levels of STAT5 activation. Knockdown of CISH in these cells resulted in increased STAT5 activation and enhanced production of DC precursors but impaired maturation of type 1 DCs, which showed reduced ability to stimulate cytotoxic T cells (249). Similarly, in the absence of CISH, GM-CSF stimulated pathological myeloid cell driven inflammation through enhanced responses to inflammatory cytokines while IL-3 stimulated increased myeloid progenitors (50).

CISH has also been implicated in the control of eosinophil function through regulation of IL-13 and IL-5 signaling. CISH was induced by IL-13 in lung fibroblasts *via* STAT6, with CISH ablation leading to increased expression of chemokines such as CCL26 in response to IL-13 to enhance eosinophil migration to the airways (260). Moreover, airway eosinophils expressed CISH at a higher level compared to peripheral blood eosinophils, which correlated with their reduced sensitivity to IL-5 signaling *via* STAT5 (261), with bone marrow cells from CISH$^{-/-}$ mice producing more eosinophils in response to IL-5 (50). It is likely that these effects on eosinophils contributes to the progressive pulmonary disease observed in CISH$^{-/-}$ mice.

CISH has been shown to impact on several models of disease. CISH$^{-/-}$ mice succumbed to experimental allergic asthma at a higher rate than their wildtype counterparts due to enhanced airway inflammation (51). Inflammation was also increased during inflammatory arthritis as well during EAE in CISH$^{-/-}$ mice (50), which also showed resistance to metastasis of tumor cells both *in vivo* and *in vitro* (49). Finally, a variety of CISH single nucleotide polymorphisms (SNPs) have been associated with increased susceptibility to and/or morbidity from a variety of infectious agents. In particular, CISH SNP-292 (rs414171) has been frequently associated with an increase in susceptibility to pathogens such as hepatitis B virus, malaria and tuberculosis infections (262–264). This SNP reduces the expression of CISH in response to IL-2 and is presumed to result in enhanced IL-2 signaling (264). However, how this results in reduced immunity to such a broad range of pathogens remains to be determined.

Other SOCS Proteins

SOCS4–7 have more pronounced roles outside cytokine signaling (7), particularly in the regulation of receptor tyrosine kinases that mediated the effects of hormones such as insulin and growth factors like epidermal growth factor (EGF) (7).

A specific role for SOCS4 in immune cell development has not been identified, with SOCS4$^{-/-}$ mice showing no significant alterations in steady-state immune cell populations (265). However, these mice were hypersusceptible to influenza virus infection, with delayed viral clearance, impaired trafficking of influenza virus-specific CD8+ T cells and a pathological increase in pro-inflammatory cytokine such as IL-1β and TNF-α, which resulted in early lethality compared to wild-type counterparts (265). The viral susceptibility was shown to be mediated by cells derived from the hematopoietic compartment (265). However, the SOCS4$^{-/-}$ mice showed no difference in CD8+ memory T cell generation or their efficient recall during subsequent infection (266). Furthermore, a recombinant herpes simplex virus (HSV) expressing SOCS4 caused reduced morbidity and mortality compared to standard HSV, which was associated with reduced levels of pro-inflammatory cytokines released mostly by macrophages during the initial innate immune response (267). This suggests that SOCS4 negatively regulates the innate immune response to viral infection to limit the resultant inflammation, although the mechanism of action remains unclear.

SOCS5 has been shown to be constitutively expressed in both B and T lymphocytes and demonstrated to regulate IL-4 signaling *in vitro* (268). However, SOCS5$^{-/-}$ mice do not display any significant abnormalities in their lymphoid compartments including their CD4+/CD8+ ratio (269). Despite this, SOCS5$^{-/-}$ mice showed heightened susceptibility to influenza virus infection, with increased viral titres and heightened pro-inflammatory cytokines particularly IL-6 and G-CSF, resulting in increased neutrophils and enhanced weight loss (270). This was mediated predominantly through non-hematopoietic tissue, with SOCS5 expression in airway epithelial cells increased following influenza infection. SOCS5$^{-/-}$ mice showed hallmarks of enhanced EGF signaling, with SOCS5 shown to be able to target both EGF receptor and the downstream PI3-K subunits, leading to enhanced Akt and STAT3 activation (270). SOCS5 levels have been found be decreased in patients suffering from chronic obstructive pulmonary disorder (COPD) (270), associated with enhanced levels of pro-inflammatory signaling through cytokines such as IL-1β and TNF-α (271). Finally, in a mouse model of allergic conjunctivitis constitutive expression of SOCS5 reduced eosinophil infiltration, possibly due to enhanced IL-4 signaling (268).

There is no evidence of SOCS6 being involved in immune cell regulation, with SOCS6$^{-/-}$ mice not showing any significant abnormalities within their immune system (272). However, there is some evidence that SOCS7 might contribute to immune cell control, since SOCS7$^{-/-}$ mice displayed different immune-related phenotypes dependent on their background. On a C57BL/6 background, SOCS7$^{-/-}$ mice showed a minor increase in neutrophils with around half succumbing to hydrocephalus of unknown etiology (273). In contrast, around half of SOCS7$^{-/-}$ mice on a 129/Sv background developed a severe cutaneous disease characterized by increased mast cell activation, upregulation of IgE and IgG, and mast cells showing increased sensitivity to IgE-induced pro-inflammatory cytokines (274). The signaling pathways involved remained to be identified.

THERAPEUTIC APPLICATIONS

Since SOCS proteins play significant roles in the coordination of the immune system, with functions identified in inflammation and immune-related malignancies, their modulation has clear potential for therapeutic intervention. As such, SOCS proteins in various conformations have been developed with some showing promise in pure clinical models. For example a mimetic peptide of the SOCS1 KIR, Tkip has produced efficacious results in the murine EAE model, with inflammatory phenotypes reduced upon administration (275). Similar to the naturally occurring SOCS1, Tkip was found to inhibit IFN-γ signaling and thereby suppress the effector functions of T-cells, ultimately compensating for the low levels of SOCS1 and SOCS3 associated with EAE in mice (275). A different mimetic peptide of SOCS1, R9-SOCS1-KIR was also able to suppress the development of experimental autoimmune uveitis (EAU) in mice with topical administration suppressing the inflammatory activities of IFN-γ, TNF-α, and IL-17, thereby protecting the mice from ocular pathologies (276). Other SOCS1 mimetic peptides have also shown anti-inflammatory effects in mouse models of chronic intraocular inflammatory disease by suppressing the effects of pro-inflammatory cytokines toward retinal cells (277). A cell penetrating SOCS1 (CP-SOCS1) has been found to suppress the induction of pro-inflammatory cytokines by blocking IFN-γ signaling (278). Similar to the endogenous SOCS1, CP-SOCS1 inhibited IFN-γ mediated STAT1 phosphorylation by interacting with IFN-γ pathway components such as JAK2, with the extent of inhibition being dose dependent (278) A cell penetrating SOCS3 (CP-SOCS3) was also developed in order to treat acute liver injury in mice caused by excessive signaling through TNF-α and IFN-γ and found to suppress inflammation driven by

inducers such as LPS, limiting the necrosis and apoptosis in the liver (279). CP-SOCS3 was also found to act in a similar manner to its endogenous proteins *in vitro,* although it retained its inhibitory effects for a much longer period of time compared to endogenous SOCS3 (280). Furthermore, deletion of the SOCS box domain in both CP-SOCS1 and CP-SOCS3 further extended their activities and half-life, providing a much more robust anti-inflammatory affect compared to their endogenous counterparts (278, 280).

SOCS proteins have also been shown to impact on immune responses relevant to inflammatory diseases and cancer, which has seen applications to immunotherapy approaches. For example, adoptive transfer of SOCS3-deficient DCs reduced the severity EAE (200), while in mice models of asthma this only slightly alleviated the development of immunogenic tolerance (281). Alternatively, adoptive transfer of SOCS3 deficient macrophages was found to exacerbate the development of acute lung injury in mice, emphasizing the multifaceted roles of SOCS3 (220). Lastly, modulation of CISH was found to be effective in protecting against tumor subtypes under the control of NK cells (282). SOCS1 ablation in human DCs was shown to be required to break self-tolerance in order to induce anti-tumor responses (283), resulting in enhanced activation of DCs, increased IFN-γ and enhanced killing by cytotoxic T cells (284, 285). In addition, treatment with tumor cell-conditioned media was shown to upregulate SOCS1 to suppress DC maturation (286). Furthermore, SOCS1 silencing in macrophages also suppressed tumor development due to enhanced anti-tumor inflammation (287). In contrast, adenovirus delivery of SOCS1 was shown to enhance T cell-mediated anti-tumor immunity (288). Combination therapies involving CISH inhibition, and BRAF as well as MEK inhibitors can extend the anti-tumor effects to additional subtypes, providing additionally opportunities for CISH modulation (282).

CONCLUSION

It is evident that SOCS proteins represent an important intrinsic mechanism for maintaining homeostasis especially within the immune system. As such it is unavoidable that their dysregulation significantly affects the delicate and complex processes that govern immunity leading to a variety of diseases particular inflammation, autoimmunity and cancer. Hence, it is vital that the various pathways regulated by SOCS proteins be identified to better understand the conditions caused due to their dysregulation. While linear pathways involving one cytokine and SOCS protein are now well-understood, the complex interplay between multiple pathways which ultimately controls cellular responses awaits considerable further attention. Moreover, it is important to look beyond the initial notions that the influence of SOCS proteins is merely inhibitory as recent evidence suggests that SOCS proteins can also have a stimulatory effect on some pathways. Moreover, the vast majority of studies regarding SOCS proteins have focused on CISH and SOCS1–3, while SOCS4–7 have only been studied in a handful of papers. While CISH and SOCS1–3 are clearly more important with regard to the immune system, recent studies have shown that SOCS4–7 likely also contribute, warranting additional studies on these proteins. Lastly, the targeting of SOCS proteins for therapeutic purposes continues to show promise. Therefore, there remains much work to do to fully understand the SOCS proteins and harness them to fight disease.

AUTHOR CONTRIBUTIONS

MS and AW contributed to the conception of the review. MS wrote the first draft of the manuscript and prepared the initial figures with assistance from CL. AW contributed additional material for the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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BRIEF RESEARCH REPORT
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Gene Expression Analysis in Four Dogs With Canine Pemphigus Clinical Subtypes Reveals B Cell Signatures and Immune Activation Pathways Similar to Human Disease

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Pemphigus is a group of autoimmune-mediated mucocutaneous blistering diseases characterized by acantholysis. Pemphigus has also been recognized in dogs and shares similar clinical characteristics and variants with human pemphigus. While relationships between human and canine pemphigus have been reported, gene expression patterns across species have not been described in the literature. We sought to perform gene expression analysis of lesional skin tissue from four dogs with various forms of pemphigus to examine gene expression during spontaneous disease in dogs. We found increased T and B cell signatures in canine pemphigus lesions compared to controls, as well as significant upregulation of *CCL3*, *CCL4*, *CXCL10,* and *CXCL8 (IL8)*, among other genes. Similar chemokine/cytokine expression patterns and immune infiltrates have been reported in humans, suggesting that these genes play a role in spontaneous disease. Direct comparison of our dataset to previously published human pemphigus datasets revealed five conserved differentially expressed genes: *CD19, WIF1, CXCL10, CD86,* and *S100A12*. Our data expands our understanding of pemphigus and facilitates identification of biomarkers for prediction of disease prognosis and treatment response, which may be useful for future veterinary and human clinical trials.

Keywords: canine (dog), pemphigus, autoimmune blistering diseases, gene expression, NanoString, cytokine, skin, comparative immunology

INTRODUCTION

Pemphigus is a group of potentially life-threatening autoimmune blistering diseases characterized by acantholysis. In these diseases, IgG autoantibodies target intraepithelial adhesion molecules resulting in epidermal splitting and blister formation (1). The incidence of pemphigus varies substantially around the world and is estimated to range between 0.75 and 5 new cases per million (2, 3).

Pemphigus can be categorized into different subtypes including pemphigus vulgaris and pemphigus foliaceus, depending on the level of blistering in the epidermis. In pemphigus foliaceous, the disease is restricted to the upper part of the epidermis, whereas in pemphigus vulgaris,

deeper layers of the epidermis are involved resulting in a more erosive presentation (4, 5). In the United States, pemphigus vulgaris is the most common subtype, primarily affecting women between 50 and 60 years of age (2, 6). Pemphigus vulgaris is thought to result from autoantibody action against desmosomal components, primarily desmoglein (Dsg) 1 and Dsg 3, leading to acantholysis (7).

Pemphigus has also been recognized in dogs and shares similar clinical characteristics and variants with human pemphigus (8). While pemphigus vulgaris is the most prevalent subtype of pemphigus in humans, it is uncommon in dogs (9). Pemphigus foliaceus is believed to be the most common variant in dogs (9). It is most often caused by autoantibodies targeting desmocollin-1 (10), which is in contrast to human pemphigus foliaceus disease, in which desmoglein-1 (DSG1) represents the major autoimmune target (1, 11). In canine pemphigus foliaceus, but less commonly in humans, transient pustules develop, which rapidly evolve into yellowish crusts and erosions due to neutrophilic infiltration that precedes acantholysis (8, 12). In dogs, lesions are often localized to the face or footpad, and later progress into generalized distribution. Pemphigus erythematosus is considered a variant of pemphigus foliaceus, with overlapping immunologic and clinical features of both lupus and pemphigus entities (13). Canine pemphigus erythematous lesions are characterized by acantholytic pustules along with a lichenoid interface dermatitis that is commonly restricted to the face.

While similarities between human and canine pemphigus have been reported, gene expression patterns across species have not been described in the literature. An advantage of studying spontaneous pemphigus in dogs is that it eliminates the layer of potential bias inherent in constructing an inducible animal model. We analyzed lesional skin tissue using NanoString technology from four dogs with various forms of pemphigus who presented to community veterinary clinics and were biopsied for diagnostic purposes. We present gene expression analyses from this case series, providing further evidence that canine pemphigus closely mimics the human condition. Our goal is to help identify novel target genes for further study as both biomarkers and drivers of pathogenesis.

MATERIALS AND METHODS

Clinical Samples

The animal study was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at the Tufts University Cummings School of Veterinary Medicine. Specimens from veterinary patients were collected when seen at the Cummings School of Veterinary Medicine, and stored in the biorepository after obtaining written consent from owners.

Skin biopsies demonstrating "pustular dermatitis" or "acantholytic dermatitis" between 2011 and 2019 were drawn from the biorepository, and potential cases were submitted to a veterinary dermatologist and pathologist for categorization into their appropriate diagnoses. Inclusion criteria for pemphigus samples included clinical presentation compatible with pemphigus disease, with morphological diagnoses of subcorneal, intragranular or subgranular acantholytic neutrophilic or mixed neutrophilic/eosinophilic pustules, and absence of proteolytic acantholytic dermatoses (staphylococcal and pustular dermatophyte infections of the skin) after histochemical stains (PAS and/or Gram). The different pemphigus subtypes were classified as follows:

- Pemphigus Foliaceous: Pustules rapidly evolving into shallow erosions and crusts with predominance to the face and feet. Histopathologic findings must include superficial epidermal, or follicular pustules rich in neutrophils with clustered acantholytic keratinocytes. Other acantholytic neutrophilic pustular diseases must be ruled out.
- Pemphigus Vulgaris: A clinical presentation that includes mucosal and/or cutaneous vesicles, bullae, deep erosions, or ulcers. Histopathology must show suprabasal epidermal, mucosal, or follicular acantholysis.
- Pemphigus Erythematosus: Pustules, erosions, and crusts localized to the face and pinnae, along with depigmentation, erythema, and erosion/ulceration of the nasal planum and dorsal muzzle. Histology morphological diagnosis should reveal intragranular to subcorneal neutrophilic and eosinophilic acantholytic pustules with a lichenoid interface dermatitis.

Immunologic tests were not performed on these animals; therefore, diagnoses were based principally on clinico-pathological grounds in accordance with Olivry et al. (8).

Control tissue were also obtained from the biorepository. These tissue samples were derived from canine healthy margin skin from leg amputations. The surgeons routinely excise an extra margin of tissue to look for malignancy, necrosis, etc. Histopathologic data from all cases were reexamined and validated by a board-certified veterinary pathologist and dermatologist (NAR, RMA). A summary of the clinical characteristics is included in the Case Presentations section.

Isolation of RNA From Formalin-Fixed, Paraffin-Embedded (FFPE) Blocks

FFPE blocks were sectioned into 30 μm thick curls and stored in Eppendorf tubes at ambient temperature. RNA extraction and purification from FFPE blocks was conducted using the Qiagen FFPE RNeasy kit, according to manufacturer's instructions. Briefly, razor blades were treated with RNase, excess paraffin was trimmed, and tissues were sliced into thin 5 μm section before treatment with deparaffinization solution (Qiagen). RNA was assessed for quantity using a NanoDrop Spectrophotometer (NanoDrop Technologies, USA), which measures RNA concentration by calculating UV absorbance.

Nanostring Cartridge, Processing, and Analysis

We quantified gene expression from canine pemphigus patients using a custom NanoString canine gene panel comprised of 160 genes involved in various immune pathways and skin homeostasis. We used *B2m, Rpl13a, cg14980*, and *hprt* as housekeeping genes. RNA hybridization was carried out using a BioRad C1000 touch machine. The hybridized samples were then

loaded into NanoString cartridges and analyzed with a Sprint profiler. Data analysis was performed using nSolver software (14) and raw counts and DEGs were plotted using GraphPad Prism v9. Raw data are shared on Gene Expression Omnibus (GEO) Database under accession #GSE171079.

H&E and IHC

Biopsy samples were formalin fixed and paraffin wax-embedded. Five-micron sections were either stained with haematoxylin and eosin (H&E) on an automated slide stainer (Sakura Tissue-Tek DRS), or stained for immunohistochemistry using rabbit anti-canine CXCL10 (US Biological, 1:100) using an automated staining machine (Dako EnVision + Dual Link System-HRP). Staining was visualized using a HRP conjugated secondary anti-rabbit antibody. Images were captured using an Olympus BX51 microscope equipped with Nikon NIS Elements software version 3.10.

Canine and Human Dataset Comparison

To determine the number of conserved genes between human and canine pemphigus, we compared our dog NanoString dataset to a human pemphigus microarray dataset available on GEO database [GSE53873; (14)]. We first found the common denominator genes from both datasets using the "MATCH" function in Microsoft Excel, then truncated the datasets to reflect shared transcripts covered in each study (total 156 genes). We next examined the differentially expressed genes (DEGs) in each dataset with $p < 0.05$. To determine total overlap of significant DEGs shared by both human and canine pemphigus disease, we compared the DEG lists of both species and generated a Venn diagram using the BioVenn web application (15).

Statistics

We performed statistical analysis to compare healthy to pemphigus lesional skin using nSolver software (NanoString, Seattle, WA) and GraphPad Prism software (version 9.0, GraphPad Software, San Diego, CA). Shapiro-Wilk tests were performed to check for normality. Data were analyzed with the two-tailed student's *t*-test if data were determined to be normally distributed, or with the two-tailed Mann Whitney *U*-test for non-normally distributed data. $P < 0.05$ were considered significant, and $P < 0.01$ were considered highly significant.

CASE PRESENTATIONS

Case 1

Ten-year-old spayed female Miniature Schnauzer that presented with a 3-week history of nasal discharge and a 10-day history of right front limb lameness. On physical examination, ulcerative lesions on the left nasal planum and right paw pad were observed, along with swelling of the tongue and palate. Biopsy of the hard palate, nasal planum and tongue demonstrated mucosal hyperplasia with suprabasilar clefting, focal ulceration and mild lymphoplasmacytic interface inflammation. Moreover, there was marked pigmentary incontinence in the nasal planum. These findings were most consistent with pemphigus vulgaris.

Case 2

A 9-year-old spayed female Labrador Cross with a history of ulcerative stomatitis presented to the emergency room due to difficulty prehending food. On physical exam, ulcerations were noted along the face, ears, and inguinal skin. The area was very sensitive to touch. A skin biopsy was taken, revealing a thickened epidermis with acanthosis. In the stratum spinosum and stratum granulosum, there were ragged and ruptured foci and clear spaces, suggestive of vesicle formation. Along the dermo-epidermal junction, there was edema, pigmentary incontinence, and moderate inflammatory interface dermatitis. These findings were consistent with pemphigus erythematosus.

Case 3

Seven-year-old female spayed German Shepherd Cross presented with a 1.5 year history of nasal and periocular depigmentation and crusting. Physical examination demonstrated thick yellow crusting on the ear flaps, around both eyes, above the nasal planum, and in multifocal areas along her flanks. She has previously responded well to steroids but has discontinued due to adverse side effects. Histopathologic evaluation showed moderate lymphoplasmacytic interface dermatitis with pigmentary incontinence. Moreover, marked eosinophilic and neutrophilic, subcorneal and exudative dermatitis with infrequent *ghost* acantholytic cells were observed. Clinical and histologic findings were consistent with pemphigus erythematosus.

Case 4

Twelve-year-old male neutered Portugese Water dog presented with a 10 month history of nasal crusting, ulceration and allergic rhinitis. On physical exam, crusting was observed on the nasal planum with erosion and depigmentation. The canine patient was initially diagnosed with mucocutaneous pyoderma, however his condition was unresponsive to antibiotic treatments and later progressed to involve the foot pads. Histopathologic evaluation demonstrated suprabasilar neutrophilic pustules with rafts of acantholytic keratinocytes. Additionally, there was superficial dermal and interface inflammatory infiltrate composed of lymphocytes and plasma cells. These findings were consistent with pemphigus foliaceous disease.

RESULTS

Key Immune Genes Are Upregulated in Canine Pemphigus vs. Healthy Controls

To begin to characterize gene expression in canine pemphigus, we isolated RNA from archival skin biopsies from four canine pemphigus patients, and five healthy controls. We used NanoString technology to analyze RNA from these samples as it quantifies genes without the needs of amplification and is useful for measuring the gene expression of difficult samples which cannot be easily amplified in PCR. It also has a considerable fast turnaround time and offers robust data.

Utilizing the high-fidelity NanoString nCounter platform, we analyzed 160 sets of canine genes to compare gene expression patterns between pemphigus and healthy canine patients (H&E from cases presented in **Figure 1A**). Agglomerative clustering of

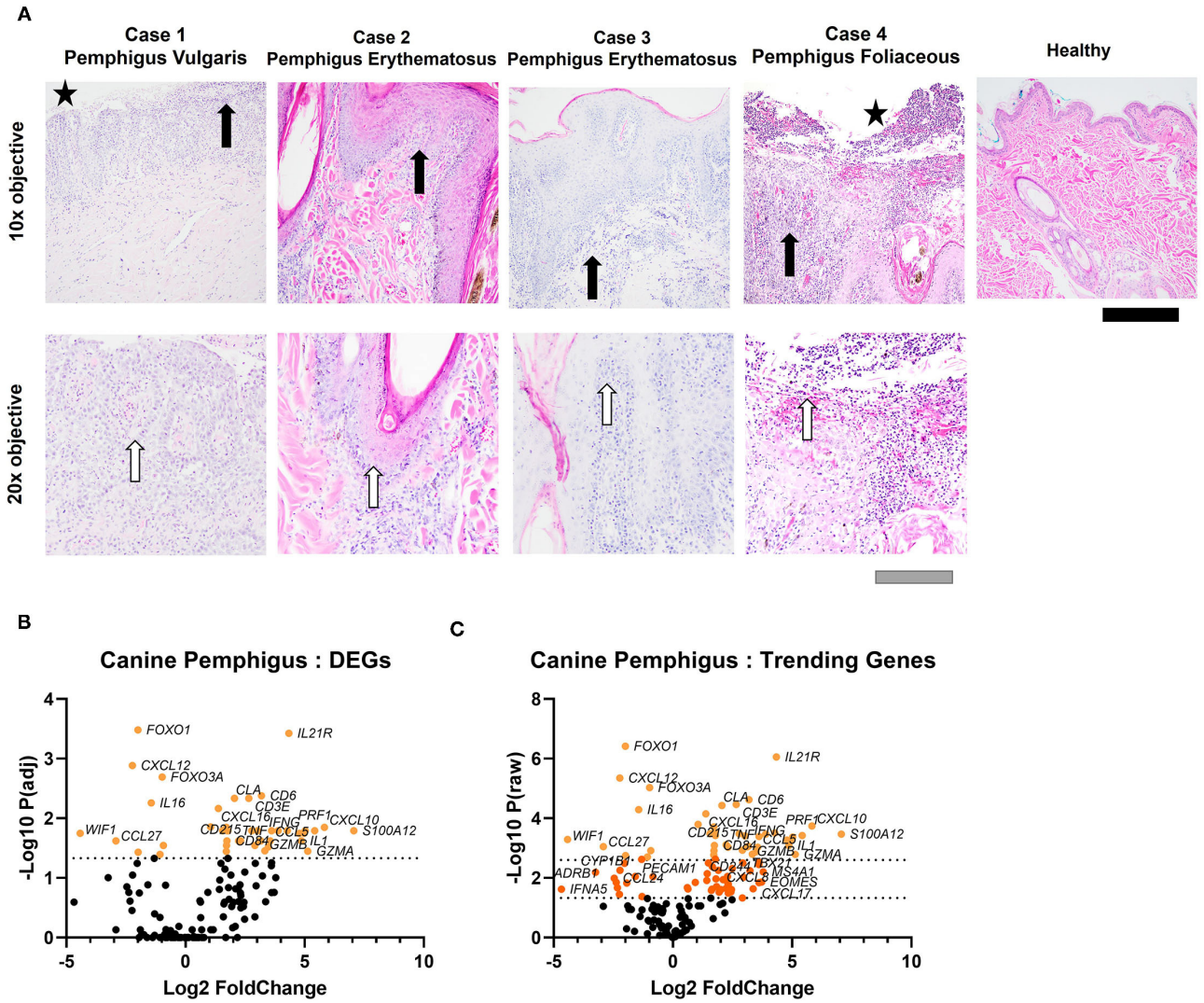

FIGURE 1 | H&E staining of canine pemphigus and healthy canine skin reveals histopathologic features of pemphigus variants. **(A)** All cases revealed interface dermatitis, edema, and pigmentary incontinence at the dermo-epidermal junction (black arrows indicate interface dermatitis). Case 1, pemphigus vulgaris, and case 4, pemphigus foliaceous, revealed epidermal sloughing (black stars). Polymorphonuclear cells were noted in all biopsies (white arrows). Scale bars black = 100 μm, gray = 200 μm. **(B)** Volcano plot of differential gene expression (DEG) analysis in canine pemphigus vs. healthy skin margins using BY adjusted *P*-values or **(C)** raw *P*-values ($n = 4$ pemphigus cases and 5 healthy margin controls).

the comprehensive set of genes discriminates between healthy and pemphigus canine skin (**Supplementary Figure 1**). Principal component analysis (PCA) showed that the transcriptomic profiles of pemphigus cases are distinguishable from healthy cases (**Supplementary Figure 2**). Out of the 160 genes, 43 genes were differentially expressed, using a Benjamini-Yekutieli (BY) adjusted $P < 0.05$ (**Figure 1B**). Nine of these genes had a negative log2 fold change (downregulated compared to healthy), and the remaining 34 genes had a positive log2 fold change (upregulated compared to healthy). We also examined genes that were trending toward significance, as these might provide insights into additional genes driving pathogenesis of canine pemphigus, but were under-powered in our study of four cases (**Figure 1C**). Ninety-four genes had a raw $P < 0.05$. Forty-six of these genes were upregulated with a log fold change of >2. Sixteen genes were downregulated with a log fold change <-1.5.

To understand the immunopathogenesis of canine pemphigus, we performed more detailed analysis on subsets of genes based on known cellular functions and previously published gene expression analyses of human pemphigus. First, we examined cytokines and chemokines. We found significant upregulation of the chemokines *CCL3*, *CCL4*, *CXCL10,* and *CXCL8 (IL8)* (**Figure 2A**). There was also significantly increased *TNF*, *IFNG*, and *IL21R* (though not *IL21* cytokine) expression compared to healthy controls (**Figure 2B**). Many of these cytokines or cytokine families have been previously reported to

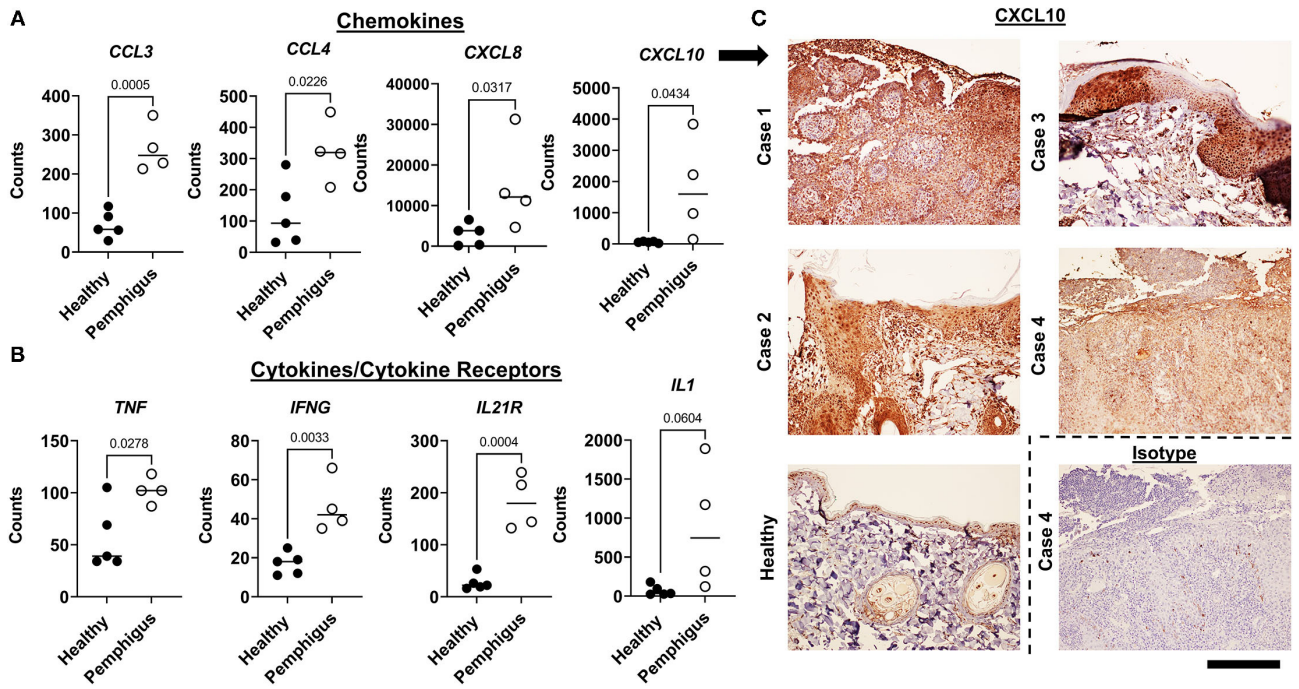

FIGURE 2 | Examination of key inflammatory gene expression in canine pemphigus variants reveals conserved patterns similar to previously published human pemphigus datasets. **(A)** Gene expression of chemokines and **(B)** cytokines/receptors in canine skin biopsies that were previously identified to be upregulated in human pemphigus [Mann-Whitney *U*-tests (non-normally distributed data) and student's two tailed *t*-tests (normally distributed data) significant as indicated; $n = 4$ pemphigus and 5 healthy margins]. **(C)** Confirmation of CXCL10 expression with IHC in cases vs. healthy and isotype controls. 100x, Scale bar = 300 μm.

be induced in blistering disorders (16). There was a trend toward increased *IL1* expression, but this was not statistically significant by two tailed student's *t*-test (**Figure 2B**).

CXCL10 Is Highly Expressed at the Protein Level in Canine Pemphigus Lesions

To confirm translation of key genes identified in our NanoString codeset, IHC was performed for CXCL10. Results showed that lesional skin biopsies from dogs demonstrated increased expression of CXCL10 compared to isotype and healthy controls (**Figure 2C**). CXCL10 was expressed throughout the epidermis and has been implicated in the pathogenesis of human pemphigus.

Skin Homeostatic Genes Are Downregulated in Canine Pemphigus

We examined genes related to skin homeostasis, as well as immune regulatory and neuroendocrine genes in our canine dataset to understand more about the skin tissue microenvironment. Analysis of neuroendocrine genes revealed that *ADRB1*, *MCR5*, and *CYP1B1* were significantly downregulated in pemphigus vs. healthy controls (**Supplementary Figure 3**). *IGFBP5*, which is known to mediate fibrosis, and *FOXO1*, which is associated with adipogenesis, were significantly downregulated in lesional skin (**Supplementary Figure 3**). Several other skin homeostatic genes, including *WIF1*, *EDA*, *RXRG*, and *TGFB2*, were significantly decreased in pemphigus cases. The keratinocyte differentiation marker, involucrin *IVL*, was significantly enriched in pemphigus lesions. *KRT14*, a marker of keratinocytes, was significantly higher, and *PECAM1*, a marker of endothelial cells, was significantly lower in pemphigus cases vs. controls (**Supplementary Figure 3**).

Cell Type Analysis Reveals Infiltration of Activated Lymphocytes and Granulocytes in Canine Pemphigus Lesions

There was a significant increase of *CD45+* immune cells in the skin of canine pemphigus patients compared to controls (**Figure 3A**). To begin to characterize this inflammatory infiltrate, we used NanoString Advanced Analysis, a method that has been previously validated with flow cytometry, to determine which cellular gene signatures were present in canine pemphigus (17) (**Figure 3B**). Cell type enrichment analysis revealed that T cells, including cytotoxic T cells are highly significantly increased in canine pemphigus compared to healthy controls, with a log2 cell type enrichment score of $P < 0.01$ (**Figure 3C**). We examined expression of the transcription factor *FOXP3*, which serves as important master regulator of regulatory T cell (Treg) differentiation and found that it was upregulated in pemphigus compared to controls (**Figure 3C**). Mast cells, B cells, and Neutrophils each had a log2 cell type enrichment score of $P = 0.05$, which means their presence in tissue has a moderate

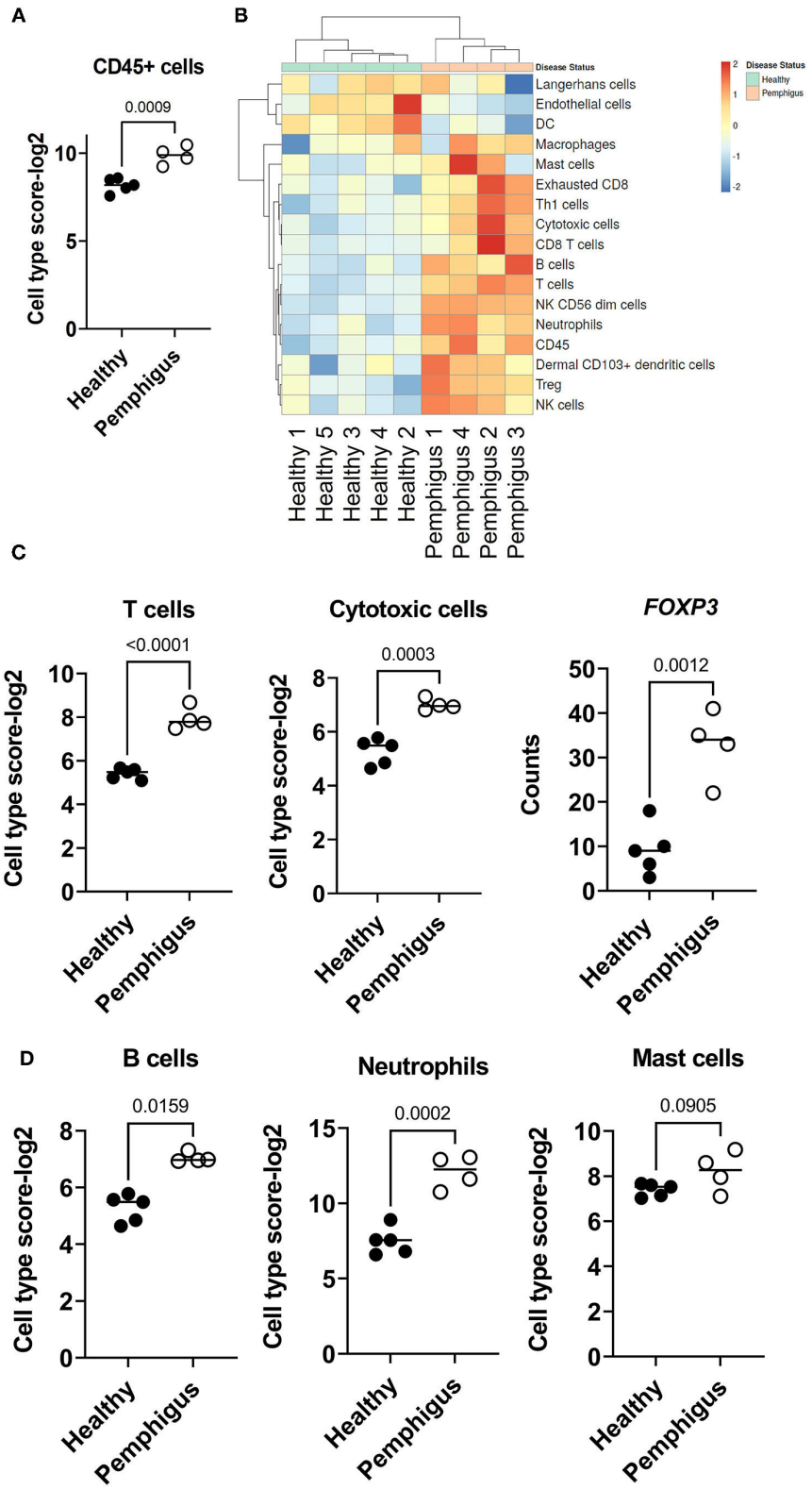

FIGURE 3 | Cell type enrichment scores in canine pemphigus reveals infiltrations of T, B, NK cells, and neutrophils. **(A)** Total CD45+ infiltrates were significantly higher in cases vs. healthy margins. **(B)** Heatmap of cell type scores in healthy (green) and pemphigus (orange) samples. **(C)** Cell type scores from NanoString advanced

(Continued)

FIGURE 3 | analysis with high confidence of prediction ($P < 0.01$) demonstrating significant increases in T cells (*CD3E, SH2D1A, CD6, TRAT1* composite score), cytotoxic cells (*GZMA, KLRK1, PRF1, GZMB, KLRB1, KLRD1, CTSW* composite score), and Tregs (*FOXP3*) in canine pemphigus skin compared to healthy controls. **(D)** B cell (*CD19, MS4A1, BLK, FCRL2, TNFRSF1* composite score), neutrophil (*CEACAM1, S100A12, CSF3R* composite score) and Mast cell (*HDC, MS4A2, CPA3* composite score) cell type analysis scores had $P < 0.05$, indicating moderate confidence for prediction. B cells and neutrophils were significantly increased in canine pemphigus compared to healthy controls ($n = 4$ pemphigus cases and 5 healthy controls, two tailed student's *t*-tests significant as indicated).

accuracy for prediction. B cells and neutrophils were increased in canine pemphigus skin (**Figure 3D**).

Comparative Analysis of Canine and Human Pemphigus Reveals Shared Differentially Expressed Genes

We next examined shared gene expression patterns between human and canine pemphigus. We selected all untreated samples from dataset GSE53873 (16), which included skin biopsies from human pemphigus vulgaris and foliaceous patients with both localized and generalized disease, as our four canine cases also exhibited clinical heterogeneity. We first truncated both datasets to a list of common denominator gene transcripts that were captured in both platforms, which yielded a list of 156 genes. Next, we examined which of these common denominator genes were differentially expressed in each species, and then used BioVenn software to compare the significant DEGs for human and canine. Our analysis demonstrated five DEGs that are conserved in all clinical subtypes of human and canine pemphigus disease: *CD19, WIF1, CXCL10, CD86,* and *S100A12*. *CYP1B1* and *PECAM1*, which were trending toward significance in our canine dataset using raw $P < 0.05$ from differential expression analysis, were significant DEGs in the human dataset. Differentially expressed genes in humans and canine pemphigus are summarized in **Figure 4A**.

To determine if any clinical subtypes share common genes, we next used the human pemphigus subtype DEGs from GSE53873 generated using the GEO2R Venn Diagram function, and we determined which, if any, DEGs were contained in the canine codeset with $P < 0.05$. We found that genes enriched in localized human pemphigus foliaceous compared to pemphigus vulgaris, including *CCL5* and *CYP1B1* were also captured in the canine DEGs when examined by two-tailed student's *t*-tests (**Figure 4B**). *TAC1* (substance *P* gene) was enriched in human pemphigus vulgaris compared to generalized pemphigus foliaceous, and this was also a DEG in our canine dataset. Generalized human pemphigus appears to be the least similar to canine pemphigus variants, though this may be due to our limited sample size or the clinical subtypes we examined.

DISCUSSION

The goals of our study were to examine immune and skin gene expression in canine pemphigus to (1) better map immunopathogenesis in veterinary patients and (2) compare to human pemphigus to understand conserved drivers of disease. Our data showed that 94 out of the 160 quantified genes demonstrated statistically significant altered mRNA expression between pemphigus and healthy canine skin. The functions of these genes vary but include both immunological and skin barrier function. We found increased expression of genes encoding T and B cell associated cytokines, chemokines, neutrophil attractants, and immune mediators.

Pemphigus is caused by autoantibodies directed against self-proteins in the skin. These autoantibodies are thought to be generated by autoreactive B cells following T cell help. As in human pemphigus, we observed both CD3+ T cells and CD19+ B cells are increased in canine pemphigus lesions (18, 19). It has been suggested that pemphigus is a T helper (Th) type 2-dependent disorder (20, 21). Other T-cell subsets, including CD4+CD25+ regulatory T cells and Th17 cells, have also been implicated in the pathogenesis of pemphigus (22, 23). *FOXO1*, a known immunoregulatory gene, was decreased in our canine cases compared to healthy controls, which may indicate reduced ability of Tregs to function to suppress autoreactive T cells in pemphigus lesions. T follicular helper cells (Tfh) have been recently reported to be critically involved in the pathogenesis of pemphigus by facilitating B-cell activation and promoting autoantibody production (24, 25). Further studies are needed to confirm if similar T-cell subset patterns exist in canine pemphigus patients.

Additionally, we noted a trend toward increased mast cells in canine pemphigus skin lesions. Increased numbers of mast cells have been detected in a prior study in the skin of pemphigus vulgaris and bullous pemphigoid patients (26–28). While the role of mast cells in the pathogenesis of pemphigus is not entirely clear, it has been suggested that mast cells may contribute to acantholysis by inducing release of esterases. Moreover, high concentrations of Dsg3-reactive IgE and intercellular IgE deposits in pemphigus vulgaris patients have been reported, further supporting the involvement of mast cells in pemphigus (29).

Recent studies have suggested a critical role for IL-17 in the pathogenesis of human pemphigus disease (19, 24). IL-17 is produced by Th17 cells and is commonly associated with the development of autoimmune conditions (30–32). While we noted increased mRNA expression of *IL17* in only one canine pemphigus case (case 4), we did note increased *IL21R*. IL-21 cytokine is directly associated with the Th17 pathway, and prior studies have demonstrated increased levels of IL-21 in human pemphigus disease. IL-21 is a cytokine secreted mainly by Tfh and Th17 cells and has been suggested to stimulate B-cell activation and differentiation, leading to pathogenic autoantibody production in pemphigus lesions (19, 33). It is possible that the signal of *IL17* and/or *IL21* from T cells is diluted by analyzing bulk mRNA from skin tissue. Therefore, future studies immunophenotyping canine pemphigus T cells by flow

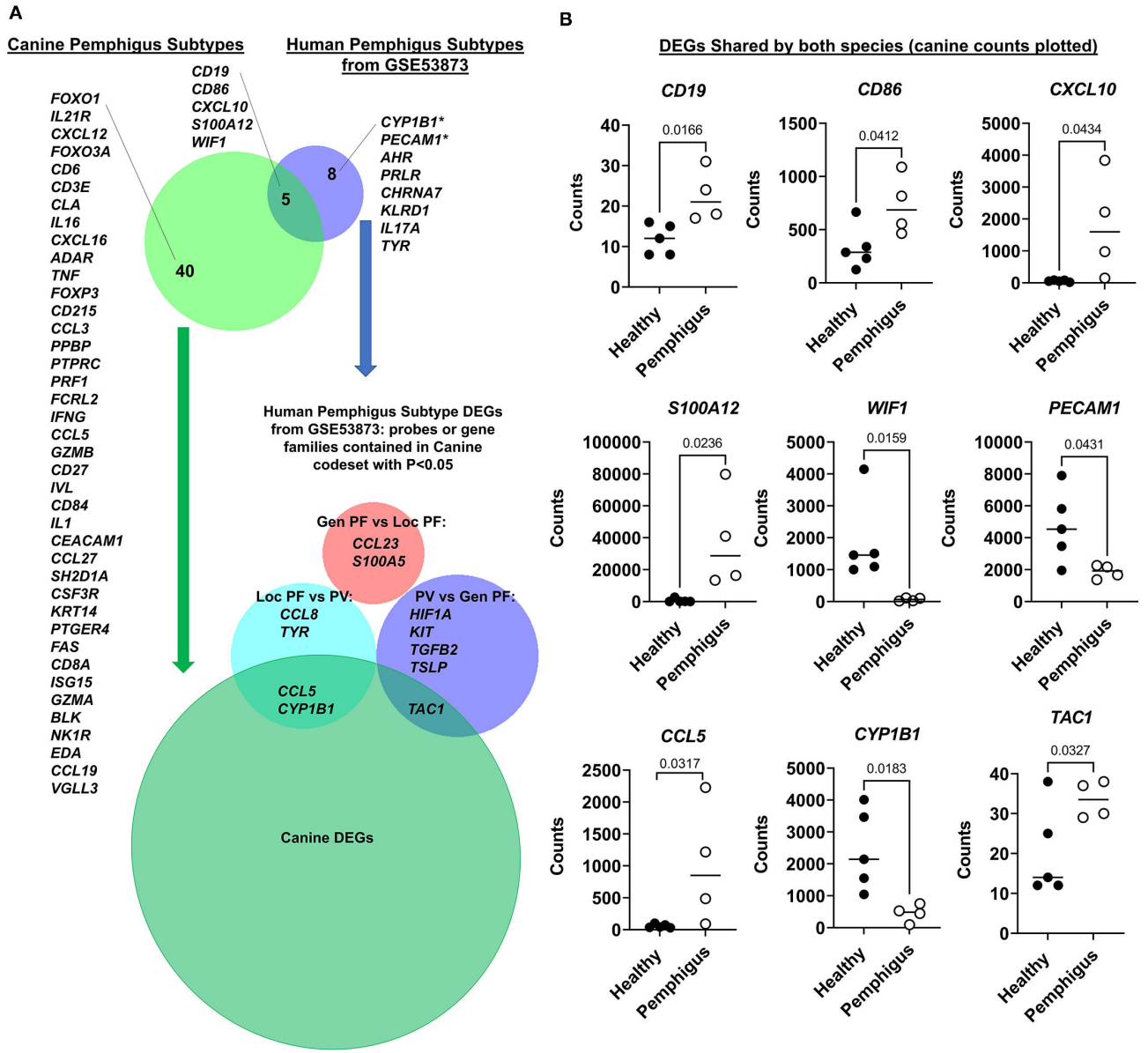

FIGURE 4 | Comparative analysis of human pemphigus cases from GSE53873 and canine pemphigus cases reveals shared DEGs. **(A)** Analysis of DEGs from pooled, untreated human pemphigus subtypes from GSE53873 and canine pemphigus subtypes. Further analysis of DEGs between human pemphigus subtypes as determined using GEO2R Venn Diagram function revealed that canine pemphigus captures elements of both human localized pemphigus foliaceous and pemphigus vulgaris, but not generalized pemphigus foliaceous. **(B)** Canine RNA counts for DEGs shared by both species are presented, including *CYP1B1* and *PECAM1*, which were trending toward significance in the overall canine differential expression analysis. **CYP1B1* and *PECAM1* are significant when directly compared between canine pemphigus and healthy margins using two-tailed student's *t*-tests (Gen PF, generalized pemphigus foliaceous; Loc PF, localized pemphigus foliaceous; PV, pemphigus vulgaris; DEGs, differentially expressed genes).

cytometry, single cell RNA sequencing, spatial transcriptomics or similar enrichment approaches is warranted.

We also found significant upregulation of chemokines *CXCL8* and *CXCL10* compared with the normal skin controls. *CXCL8* and *CXCL10* mediate recruitment of leukocytes to the skin from the blood (34, 35), and have been implicated in the pathogenesis of human pemphigus (36, 37). CXCL10 has been shown to be highly expressed in human bullous pemphigoid disease, and is suggested to induce matrix metalloproteinase 9 (MMP), which is a key proteinase in the pathologic process associated with blister formation (36). Similarly, *CXCL8* has been hypothesized to recruit neutrophils to the epidermis and contribute to the blistering process (38).

Consistent with human studies (37, 39), *TNF* was also upregulated in pemphigus lesional skin. TNF functions to regulate acute phase proteins and induces secretion of several

cytokines and chemokines (37). It also stimulates T-cells, modulates differentiation of B cells, and promotes endothelial cells to secrete neutrophil chemotactic factor to increase neutrophil migration into tissues (40–42). Thus, TNF may play a mediator role in pemphigus lesions by increasing epithelial damage.

We also examined skin-specific genes that are associated with skin biology. We found that lesional skin from dogs with pemphigus exhibited downregulation of genes involved in skin homeostasis and immune tolerance. We observed decreased *WIF-1* expression in pemphigus skin which was conserved across both canine and human datasets. The Wnt proteins have been shown to have a fundamental role in tissue homeostasis, including development of hair follicles and initiation of the anagen phase (43). Impaired Wnt signaling has been reported in psoriatic skin disease (44).

Limitations in our study include the small sample size, and number of genes analyzed (160 genes). Future studies should include larger scale comparative analyses and whole transcriptome sequencing to allow for a better understanding of the pathogenesis of pemphigus across species. Despite these limitations, we observed many differentially expressed genes that match previously published literature regarding potential drivers of pemphigus immunopathogenesis. To our knowledge, this is the first study that provides gene expression analysis for canine pemphigus immune and skin transcripts. We have shown that dogs capture features of human disease, which may benefit patient care by providing additional predictive models for human disease mechanisms. In light of the fact that CAR-T clinical veterinary and human trials are in development, we hope that our study will add potential biomarkers that could mutually benefit both species.

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 below: https://www.ncbi.nlm.nih.gov/geo/, GSE171079.

ETHICS STATEMENT

The animal study was reviewed and approved by Tufts Cummings School of Veterinary Medicine IACUC. Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

JR: conceptualization, project administration, and funding acquisition. NR and JR: methodology and resources. CD: software. RA and CP-M: validation. HR, NW, and JR: formal analysis. RA, CP-M, NR, DM, JR, and CD: investigation. NR, CP-M, RA, and JR: data curation. HR: writing–original draft. HR, CP-M, NW, and JR: visualization. JR, NR, and RA: supervision. All authors writing–review and editing.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2021.723982/full#supplementary-material

Supplementary Figure 1 | Heatmap of all samples. Rows are centered; unit variance scaling is applied to rows. Both rows and columns are clustered using correlation distance and average linkage. 160 rows, 8 columns.

Supplementary Figure 2 | PCA plot of samples. Unit variance scaling is applied to rows; SVD with imputation is used to calculate principal components. X and Y axis show principal component 1 and principal component 2 that explain 36.8 and 29.3% of the total variance, respectively. Prediction ellipses are such that with probability 0.95, a new observation from the same group will fall inside the ellipse. $N = 8$ data points.

Supplementary Figure 3 | Skin and immune homeostatic genes are decreased in canine pemphigus compared to healthy control skin. Gene expression of skin and immune homeostatic genes in cases compared to controls (Mann-Whitney *U*-tests (non-normally distributed data) or two tailed student's *t*-tests (normally distributed data) significant as indicated; $n = 4$ pemphigus and 5 healthy margins).

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Conflict of Interest: JR is an inventor on use patents for targeting CXCR3 (0#15/851, 651) and IL15 (# 62489191) for the treatment of vitiligo. NR is an employee of bluebird bio. CD is an employee of NanoString.

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MINI REVIEW
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Human CLA$^+$ Memory T Cell and Cytokines in Psoriasis

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Psoriasis is a common inflammatory skin condition resulting from the interplay between epidermal keratinocytes and immunological cellular components. This sustained inflammation is essentially driven by pro-inflammatory cytokines with the IL-23/IL-17 axis playing a critical central role, as proved by the clinical efficacy of their blockade in patients. Among all the CD45R0$^+$ memory T cell subsets, those with special tropism for cutaneous tissues are identified by the expression of the Cutaneous Lymphocyte-associated Antigen (CLA) carbohydrate on their surface, that is induced during T cell maturation particularly in the skin-draining lymph nodes. Because of their ability to recirculate between the skin and blood, circulating CLA$^+$ memory T cells reflect the immune abnormalities found in different human cutaneous conditions, such as psoriasis. Based on this premise, studying the effect of different environmental microbial triggers and psoriatic lesional cytokines on CLA$^+$ memory T cells, in the presence of autologous epidermal cells from patients, revealed important IL-17 cytokines responses that are likely to enhance the pro-inflammatory loop underlying the development of psoriatic lesions. The goal of this mini-review is to present latest data regarding cytokines implicated in plaque and guttate psoriasis immunopathogenesis from the prism of CLA$^+$ memory T cells, that are specifically related to the cutaneous immune system.

Keywords: psoriasis, CLA$^+$ T cell, IL17A, interleukins, IL17F, IL-9, IL-15

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INTRODUCTION

The regulation of cytokine production and signaling in chronic cutaneous inflammation is influenced by genetic and environmental factors. This minireview focuses on human psoriasis, both plaque and guttate forms of the disease, and the cytokine production by human circulating CLA$^+$ memory T cells, a relevant subset of memory T cells associated to the regional cutaneous immune system. In translational research in dermatology, there is a need to develop new approaches beyond animal models and *in vitro* studies (1, 2), since the results in those models are not always translated into the clinic (3). For instance, excellent basic science results in the field of cytokines in psoriasis have been generated for IFN-α, IFN-γ, IL-20, or IL-22, but their *in vivo* neutralization with monoclonal antibodies in clinical trials did not improve psoriasis severity (4–7), indicating that those cytokines, despite their presence in the lesions, are not suitable for patients' treatment.

When studying human chronic cutaneous inflammatory diseases, an alternative approach focused on the human regional cutaneous immune system in disease would allow to focus more closely on the immunological mechanism of disease that takes place within the skin, the proper organ where the pathological process is carried out. The presence of the skin associated lymphoid tissue (SALT) was proposed by J. Wayne Streilein based on the existence of some T cells with special function related only to the skin (8). The Cutaneous Lymphocyte-associated Antigen (CLA) identifies the subset of effector memory T cells with skin tropism that recirculates between skin and blood during cutaneous inflammation and reflects immunological abnormalities present in the skin. The CLA molecule is a carbohydrate modification of the platelet selectin ligand-1 (PSGL-1) (9), which binds both endothelial cell and platelet selectins (E-selectin and P-selectin) molecules expressed on post-capillary venules in the skin. Its expression is induced on $CD45R0^+$ memory T cells at skin draining lymph nodes (10) and it is present in more than 90% of cutaneous infiltrating T cells but in <20% of T cells in other peripheral tissues. Interestingly, a proportion of CLA-expressing T cells can be also found in circulation, representing around 15% of human circulating T cells (10). Indeed, the fact that CLA^+ memory T cells recirculate between skin and blood was evidenced by action of efalizumab (a monoclonal antibody targeting the CD11a subunit of LFA-1 molecule that blocked lymphocyte extravasation toward the skin) in psoriasis (11) and atopic dermatitis (12) patients, which proved clinical improvement but also resulted in a lymphocytosis effect of CLA^+ T cells that caused relapses after treatment discontinuation (13). The recirculating capacity of CLA^+ memory T cells is key to their value in translational research, due to their ability to reflect the immune abnormalities found in numerous human cutaneous conditions such as psoriasis, atopic dermatitis, contact dermatitis, drug-induced allergic reactions, vitiligo, herpes simplex, rosacea, cutaneous T cell lymphoma, or alopecia areata (14, 15).

In order to properly study the regional cutaneous immune system in psoriasis, the use of clinical material from non-treated patients provides clear advantages as it is commented below. With this purpose, we have established a new *ex vivo* coculture model made of lymphoid and epidermal cells from the same patient (16) that integrates relevant elements of psoriasis immunopathology: skin-associated memory T cells (CLA^+ T cells) isolated from blood samples, autologous lesional epidermal cells disaggregated from biopsies from the same patient and a clinically relevant trigger of the disease and flares (such as *Streptococcus pyogenes*). When circulating CLA^+ T cells and autologous lesional epidermal cells cocultures are activated by *Streptococcus pyogenes* extract (SE), specific Th17/Th1/Th22 responses occurs, together with production of disease relevant chemokines (CXCL9, CXCL10, CXCL11), which is not the case in CLA^- T cells cocultures or in unstimulated basal conditions. Interestingly, the levels of IL-17A and other cytokines expressed by CLA^+ T cells in response to SE are highly correlated with serum anti-Streptolysin O antibody titer (ASO), that is commonly used to assess patients recent infection by *S. pyogenes* in daily clinical practice. The fact that ASO levels and CLA^+ T cells-dependent IL-17A, IFN-γ, and IL-22 production are directly associated shows how these cytokines can be measured *ex vivo* and integrated with the patient clinical features and environmental exposure to *S. pyogenes*.

In the following subsections, information on cytokines in psoriasis immunopathology is provided from the prism of T cells that are related to the cutaneous immune system. These results have been generated using clinical material (blood and skin biopsies) from non-treated psoriasis patients.

INFLUENCE OF PSORIATIC INFLAMMATORY ENVIRONMENT ON CLA-DEPENDENT IL-17A AND IL-17F PRODUCTION

Memory T cells present in the psoriatic lesion are exposed to the cytokine inflammatory environment and this may influence the effector functions of the IL-17 cytokines, that are clinically relevant mediators in psoriasis. Since peripheral CLA^+ memory T cells recirculate between skin and blood during psoriasis and reflect the immunological abnormalities present in the skin, they can be used to study the lesional inflammatory environmental cytokines. The presence of IL-15 and IL-23 is increased in lesional skin from psoriasis patients. Psoriatic epidermal keratinocytes show increased expression of both IL-15 and IL-15R (17). Similarly, injured keratinocytes but also dendritic cells have been proved to produce increased amounts of IL-23 in psoriatic lesions (18). Besides, both pro-inflammatory cytokines are linked to Th17 biology. IL-15 is known to induce IL-17 secretion by T cells and to be critical for maintaining memory Th17 cells (19), whilst IL-23 is considered the "master regulator" of Th17 cell differentiation and development. Single nucleotide polymorphisms (SNPs) associated with psoriasis have been also described for both IL-15 and IL-23 (20–22). However, despite their known connection to psoriasis pathogenesis, it was not until recently that the cooperation between these two cytokines has been addressed. Our group has shown that IL-15 and IL-23 act synergistically on cocultures of CLA^+ memory T cells and autologous epidermal cells to produce significantly increased levels of IL-17F and IL-17A in psoriasis when compared to CLA^- T cells cocultures (23). Importantly, this synergy was not observed for lesional resident memory T cells or in cocultures from healthy individuals, pointing out the relevance of circulating CLA^+ T cells in psoriasis pathogenesis. Altogether, it is a clear example of how the proinflammatory milieu in psoriatic lesions influences specifically skin-tropic memory T cells and induces a Th17 response contributing to the maintenance of the disease.

CLA^+ T CELLS IN PSORIASIS PRODUCE MORE IL-17F THAN IL-17A

The IL-17 family comprises six structurally related members (named from A to F) that act as homodimers, except for IL-17A and IL-17F that can form heterodimers together. In psoriasis, IL-17A is pointed out as the most important mediator

(24), which is supported by the effectiveness of its blockade in patients. However, its principal homolog, IL-17F, has been also proved to be increased in lesional skin and blood from psoriasis patients (25–27). From our experience, circulating CLA^+ memory T cells activated with a relevant disease trigger, such as *Streptococcus pyogenes*, and in the presence of lesional epidermal cells, secrete a higher amount of IL-17F than IL-17A *in vitro* (28, 29). Similarly, increased CLA^+ memory T cell-dependent IL-17F response was observed when stimulating the cocultures with *Candida albicans* (30) or the combination of the pro-inflammatory cytokines IL-15 and IL-23 (23). As such, CLA^+ memory T cells should be considered an important source of IL-17F in psoriasis. Because IL-17A and IL-17F homodimers and heterodimer share their signaling receptor, constituted by the IL-17RA and IL-17RC subunits, they induce similar gene expression patterns. Nonetheless, the relevance of IL-17F in psoriasis pathogenesis has been shown in patients and animal models. For example, IL-17F knock-out (KO) mice are more resistant to imiquimod-induced psoriasiform inflammation than the IL-17A KO counterparts (31). In humans, the rapid clinical efficacy achieved by brodalumab, a monoclonal antibody targeting the IL-17RA subunit (that is part of the receptor for IL-17A, IL-17F, IL-17A/F heterodimer, and IL-25) first showed the advantages of targeting beyond IL-17A (32, 33). Most importantly, the promising results of the phase 2 and phase 3 clinical trials studying the nanobody sonelokimab and the monoclonal antibody (mAb) bimekizumab, respectively, both of which target IL-17A, IL-17F, and IL-17A/F, further highlight the clinical relevance of IL-17F in psoriasis patients (34, 35).

IL-9 IS NOT PRODUCED TRANSIENTLY BY CLA^+ T CELLS IN HUMAN PSORIASIS

In humans, IL-9-secreting $CD4^+$ T cells, namely Th9, have been described as a distinct T cell population with preferential skin tropism (36). In this work, CLA^+ Th9 cells were transiently induced by *Candida albicans*-pulsed monocytes particularly but also after non-physiological polyclonal activation, supporting the association of Th9 cells to the skin regardless of their antigen specificity and using lymphocytes from healthy subjects. This transitory Th9 induction peaked just before the increase of IL-17 and IFN-γ induction, suggesting a paracrine regulation by IL-9 that was further confirmed by its own neutralization. More interestingly, a significant increase of $IL\text{-}9^+$ cells, mostly $CD3^+$ $CD4^+$ T cells, was found in lesional psoriatic skin compared to atopic dermatitis and healthy cutaneous tissue.

However, when studying the functional role of IL-9 in psoriasis patients (plaque and guttate forms) in our coculture model (29), we found that, after *S. pyogenes* activation, the kinetic of IL-9 production measured in CLA^+ T cells and epidermal cells coculture supernatants was similar to those of IL-17A and IFN-γ, progressively increasing over time and dependent on MHC class I and class II presentation, which contrasted with the transient IL-9 induction reported before (36). Interestingly, IL-9 partially enhanced SE-induced IL-17A, but not IFN-γ, secretion by CLA^+ T cells, as well as it promoted the survival of the skin-homing T cell subset in psoriasis.

Using well-defined clinical samples also allows obtaining information on the relationship of cytokines with patient clinical status. CLA^+ T cells-derived cytokines are associated with clinical features of skin diseases because they represent a subset of memory T cells that are involved in the regional cutaneous immune system. To support the relevance of our findings regarding the role of IL-9 in psoriasis, it was observed that SE-induced CLA^+ T cells-dependent IL-9 correlated with psoriasis severity (measured as Psoriasis Area Severity Index, PASI) in patients. Moreover, in guttate psoriasis, the peak in IL-9 production was found on patients having the highest ASO levels indicating higher exposure to *S. pyogenes*.

GUTTATE PSORIASIS: WHERE GENETIC AND ENVIRONMENTAL FACTORS INTERACT PRODUCING TH17 RESPONSE BY CLA^+ T CELLS

Guttate psoriasis is a form of psoriasis that is strongly associated with the genetic predisposing allele HLA-Cw6 and is commonly triggered by *Streptococcus pyogenes* throat infections (37). This situation has provided a good opportunity to study genetic and environmental factors influence on cytokine production by CLA^+ T cells in psoriasis (28). The coculture of CLA^+ T cells activated with *S. pyogenes* clearly demonstrated that non-treated guttate psoriasis patients produced significantly more IL-17A/F cytokines than IFN-γ. However, the most interesting finding was that guttate psoriasis patients carrying the HLA-Cw6 allele and/or whose flare was produced by *S. pyogenes* pharyngitis, in comparison to guttate psoriasis patients that did not fulfill those criteria, significantly produced more IL-17A/F by CLA^+ T cells. Similar dominant IL-17 response also induced genes that belong to the IL-17 transcriptome in keratinocytes such as *DEFB4, LCN2, IL-8*, but no to the IFN-γ (*CXCL9, CXCL10*, and *CXCL11*). Such influence of genetic and microbe infection on IL-17 response in guttate psoriasis patients has been clarified by analyzing CLA^+ T cells.

PLAQUE PSORIASIS: IL-17 PRODUCTION AND PATIENTS HETEROGENEITY

Chronic plaque psoriasis, or *psoriasis vulgaris,* is the most common form of psoriasis that is generally diagnosed based on the shape and location of the lesions and the persistent duration of the disease. Heterogeneity among plaque psoriasis patients has been suggested (38) but it is still not well-characterized. Nowadays, plaque psoriasis is considered a single entity in most of the clinical studies yet. However, recent results have suggested that the IL-17 cytokines responses by CLA^+ T cells may be differentially influenced by environmental factors, particularly by exposure to microbes that could favor IL-17 production in chronic plaque psoriasis.

Streptococcus pyogenes-induced IL-17 response by CLA^+ T cells in psoriasis patients is higher when patients present higher

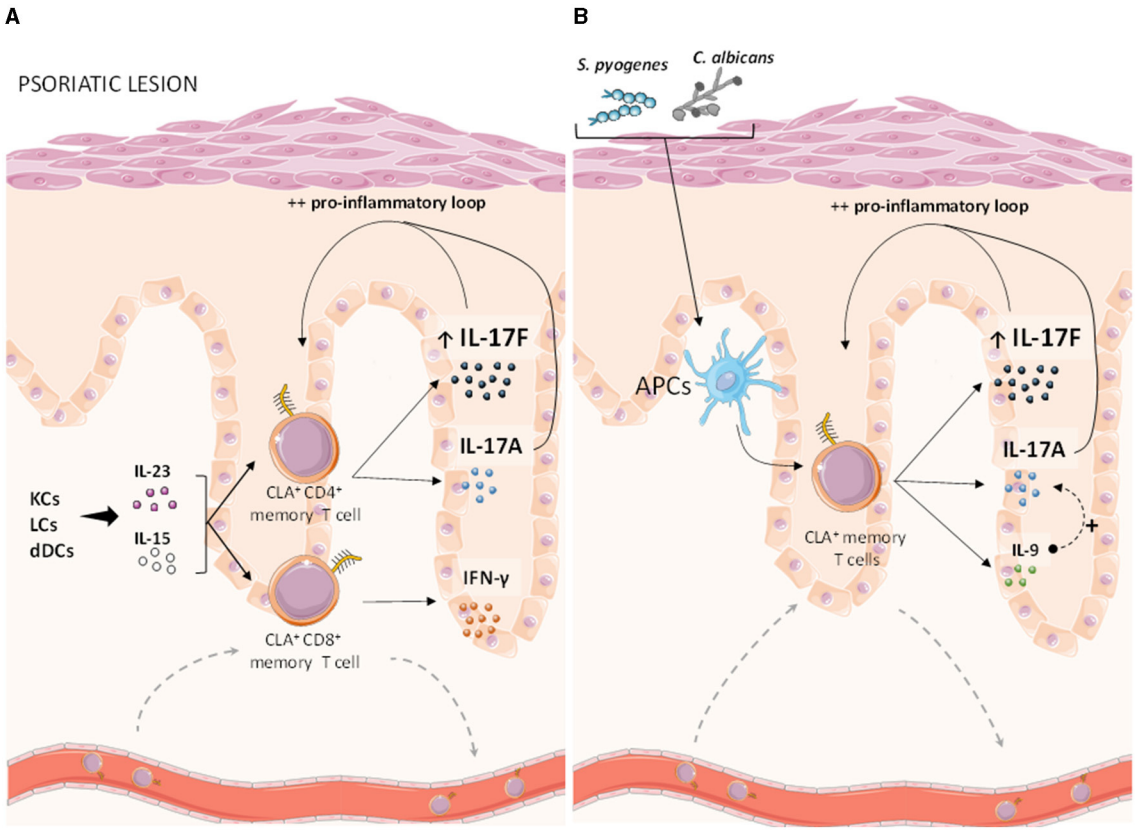

FIGURE 1 | Human CLA$^+$ memory T cells and cytokines in psoriasis. As the subset of memory T cells associated to the cutaneous immune system, circulating CLA$^+$ T cells are key responders to lesional pro-inflammatory cytokines and relevant microbes that induce important type 17 responses, therefore contributing to the pro-inflammatory loop leading to the appearance of psoriatic lesions. **(A)** IL-15 and IL-23, which are mainly produced by keratinocytes as well as epidermal and dermal dendritic cells, synergistically activate CLA$^+$ CD4$^+$ memory T cells from psoriasis patients leading to an increased induction of IL-17F and IL-17A cytokines, whereas IFN-γ secretion is likely mediated by CLA$^+$ CD8$^+$ memory T cells in psoriasis but also in healthy controls. **(B)** *S. pyogenes* and *C. albicans* antigens, presented by epidermal antigen presenting cells, have been proved to induce significantly higher IL-17A/F and IL-9 responses by CLA$^+$ memory T cells in psoriasis patients, compared to CLA$^-$ T cells. Besides, *S. pyogenes*-induced IL-9 has shown to enhance IL-17A responses, further reinforcing the pro-inflammatory loop underlying psoriasis lesions. CLA, cutaneous leukocyte-associated antigen; KCs, keratinocytes; LCs, Langerhans cells; dDCs, dermal dendritic cells; APCs, antigen presenting cells. This figure was created using Servier Medical Art (smart) images.

titer of immunoglobulin A against *S. pyogenes* extract (39). We reported that anti-SE IgA values in psoriasis patients (both plaque and guttate forms) are higher than in atopic dermatitis and healthy controls. But, most importantly, we found that increased mucosal exposure to *S. pyogenes* was present in chronic plaque psoriasis patients despite negative ASO titer and no clear association of the current flare with *Streptococcal* infection. This finding is of special interest since there may be multiple cases of plaque psoriasis where the implication of *Streptococcus pyogenes* has been discarded, but the bacteria may still be present at the tonsils and contribute to recurrent flares in those patients. In this regard, a recent population-based cohort study has reported how tonsillectomy diminished the risk of developing psoriasis (40). Future studies should confirm how such heterogeneity may influence the natural history of psoriasis disease and/or how different patients respond to specific treatments.

Moreover, chronic plaque psoriasis patients also present increased exposure to *Candida albicans* (CA) (41, 42), that is a potent inducer of IL-17 cytokines in humans (43). Importantly, immune response against this fungus is mainly mediated by CLA$^+$ memory T cells, further supporting the pro-inflammatory loop present at cutaneous lesions in psoriasis patients (30). Interestingly, non-treated chronic plaque psoriasis patients, without clinical signs of candidiasis, present increased levels of both IgA and IgG specific for *C. albicans* in plasma. In particular, CLA-dependent IL-17A and IL-17F responses correlated with *Candida*-specific IgA, but not IgG, only in chronic plaque psoriasis. Additionally, a proteomic study of plasma samples from 114 non-treated plaque psoriasis patients revealed that, those with higher anti-*Candida* IgA levels presented increased levels of proteins involved in antimicrobial humoral response, especially proteins showing anti-candida activity [such as eosinophil cationic protein (ECP/RNASE3), Chitinase-3-like protein 1 (CHI3L1) or azurocidin]. These findings point out the implication of *Candida albicans* in chronic plaque psoriasis, a matter that we believe should be further explored in the clinic.

Altogether, these results support the existence of heterogeneity among chronic plaque psoriasis patients that can influence IL-17 production and, thus, the evolution of the course of psoriasis disease.

DISCUSSION

In plaque and guttate forms psoriasis, the IL-17 response derived from CLA^+ T cells is clearly influenced by different factors that are related to patient features and that can be studied *ex vivo* using circulating memory T cells belonging to the regional cutaneous immune system (**Figure 1**). Using clinical samples from non-treated patients has allowed relating *in vitro* cytokine responses with clinical data in a translational way. For example, these studies reported increased production of IL-17F over IL-17A by skin associated T lymphocytes, which is reflected in the clinic as the greatest response of bimekizumab (neutralizing both IL-17A/F) (34). Also, this approach has shown that guttate psoriasis patients positive for the HLA-C predisposing allele and/or the ASO perform increased cutaneous pro-inflammatory response by CLA^+ T cells. In the end, how CLA^+ memory T cells are the link between relevant disease triggers and the increased IL-17 response observed in psoriatic skin.

Mouse models or complex *in vitro* systems, although they are key to study basic pathogenic mechanisms, do not integrate elements that can influence IL-17 responses in patients such as the genetic background or their exposure to microbes able to induce IL-17 responses. How these factors may influence patients response to treatment, the natural history of disease or the existence of psoriasis endotypes, if any, is still an open question in this field. In this regard, the possible role of CLA^+ memory T cells in the development of psoriatic arthritis (PsA) was early explored but discarded, due to the absence of CLA^+ memory lymphocytes within synovial tissue (44, 45). The relevance of heterogeneity in chronic plaque psoriasis due to different immunoglobulins specific for microorganisms and CLA^+ T cells-dependent IL-17A/F responses is particularly interesting since these cytokines, in contrast to others that are also increased in lesions, are validated as safe and clinically effective targets by the use of neutralizing monoclonal antibodies (46).

Nonetheless, this review presents some limitations, as other types of psoriasis (pustular, palmoplantar or inverse), have not been addressed and the study of additional relevant cytokines that are present within psoriatic lesions, such as TNF-α or IL-36, in the context of CLA^+ memory T cells remains underexplored. Eventually, these limitations evidence how much research is still needed to completely unveil the intricate between cytokines and the cutaneous immune system in the context of psoriasis. Any translational approach that helps to better characterize cytokine biology in the context of the cutaneous immune response will be essential to fully understand patients responses to current therapies and to continue developing novel strategies in the future.

AUTHOR CONTRIBUTIONS

CJ-G: writing and figure design. LS, IG-J, MF, and RP: writing. LS-B: IP project founding, writing, and manuscript conceptualization. All authors contributed to the article and approved the submitted version.

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Crosstalk Between ER Stress, Autophagy and Inflammation

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The endoplasmic reticulum (ER) is not only responsible for protein synthesis and folding but also plays a critical role in sensing cellular stress and maintaining cellular homeostasis. Upon sensing the accumulation of unfolded proteins due to perturbation in protein synthesis or folding, specific intracellular signaling pathways are activated, which are collectively termed as unfolded protein response (UPR). UPR expands the capacity of the protein folding machinery, decreases protein synthesis and enhances ER-associated protein degradation (ERAD) which degrades misfolded proteins through the proteasomes. More recent evidences suggest that UPR also amplifies cytokines-mediated inflammatory responses leading to pathogenesis of inflammatory diseases. UPR signaling also activates autophagy; a lysosome-dependent degradative pathwaythat has an extended capacity to degrade misfolded proteins and damaged ER. Thus, activation of autophagy limits inflammatory response and provides cyto-protection by attenuating ER-stress. Here we review the mechanisms that couple UPR, autophagy and cytokine-induced inflammation that can facilitate the development of novel therapeutic strategies to mitigate cellular stress and inflammation associated with various pathologies.

Keywords: autophagy, unfolded protein response, ER-stress, cytokines, inflammation

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INTRODUCTION

Inflammation is the primary defense mechanism mounted to protect the host against infection when cells sense pathogen associated molecular patterns (PAMPs) through the pattern recognition receptors (PRRs). Inflammation can also be triggered when the immune system senses substances released by damaged cells known as damage associated molecular patterns (DAMPs) which are also recognized by PRRs. Profound and chronic inflammation is damaging hence, associated with pathological conditions such as intestinal bowel disorder (IBD), chronic obstructive pulmonary disease (COPD), diabetes, obesity, cardiovascular diseases, multiple sclerosis and rheumatoid arthritis. On the other hand, there is epidemiological, clinical andexperimental evidence that inflammation can be triggered in the absence of PRR-mediated sensing and -signaling through cellular stress, wherein biological processes within cellsare impaired resulting in increased inflammation linked to the above-mentioned pathologies (1).

When cells are stressed, they trigger signaling pathways which enable the cells to adapt to the changes caused by the stress. For a cell, these perturbations can be either life-enhancing or life-threatening (2). Likewise, any chronic perturbations disturbing the endoplasmic reticulum (ER) homeostasis results in ERstress. ERstress is characterized by the accumulation of aberrant proteins which influence the protein folding capacity of the ER (3). The cell responds to ER stress by initiating unfolded protein response (UPR), which is aimed at increasing the ability

of ER to fold proteins properly, regulate protein translation and induce cell death if everything fails. Interestingly, UPR proteins also regulate inflammation associated with diseases (4). Autophagy is another process which is activated upon cellular stress to reestablish homeostasis. It aids in the lysosomal degradation of damaged organelles, denatured proteins and pathogens. Autophagy also plays a critical role in regulating inflammation by promoting the immune cell-survival and regulating the expression and secretion of inflammatory cytokines (5). Intriguingly, the UPR pathways that regulate inflammation also intersect with mechanisms that regulate autophagy and together govern the outcome of inflammatory diseases. Therefore, in this review we describe the molecular links between UPR, autophagy and inflammation and their involvement in some of inflammatory diseases.

ER STRESS AND UPR MECHANISMS

The endoplasmic reticulum (ER) is responsible for major cellular functions such as protein folding, synthesis of lipids, sterols and calcium storage (2, 6). ER function can be influenced by a wide variety of factors. For instance, hypoxia, glucose deprivation, hyperthermia, acidosis, calcium levels, altered metabolism, infections, mutations in secretory proteins and inflammation can disturb appropriate functioning of the ER, impacting protein folding (7). This causes an imbalance between the demand for protein folding and the capacity of the ER for protein folding resulting in the accumulation of misfolded/unfolded proteins (2, 6, 7). This condition is termed as "ER stress" which has been implicated in various inflammatory conditions associated with cardiovascular, neurodegenerative and metabolic diseases (8).

In response to ER stress, unfolded protein response (UPR) is activated (9, 10). UPR comprises of three distinct signal transduction arms mediated through, protein kinase RNA (PKR)-like ER kinase (PERK), inositol-requiring protein-1 (IRE1) and activating transcription factor-6 (ATF6). Each UPR activator protein consists of three domains; ER luminal domain (LD), a single membrane-spanning domain, and a cytosolic domain (CD). Under physiological conditions, PERK, IRE1 and ATF6 are bound via their LD to binding immunoglobulin (BiP) and remain in inactive state (11, 12). BiP is the most abundant Hsp70-type ER chaperone and a direct ER stress sensor (12, 13). Upon accumulation of misfolded/unfolded proteins, BiP then initiates UPR by sequestering away from the luminal domains of PERK, IRE1 and ATF6 and binds to nascent polypeptidesto chaperone proper folding andattain native conformation (13). Dissociation of BiP from IRE1, PERK and ATF6activatesthe three distinct UPR branches (14). Subsequently, UPR regulates the rate of protein synthesis, translocation of proteins into the ER, chaperoning the misfolded proteins, andprotein trafficking (15). Thus,UPR signal transducers are critical for ER quality control and maintaining ER homeostasis.

ER STRESS INDUCED AUTOPHAGY

Increased presence of misfolded/unfolded protein load in the ER is harmful to cells and therefore cells have evolved mechanisms that can detect, unfold and refold the misfolded/unfolded proteins (6). However, the misfolded proteins that cannot be repaired are eliminated from the cell through the specialized processes, ER-associated protein degradation (ERAD) and autophagy (7, 15). ERAD involves recognition of aberrant proteins by the molecular chaperones and translocation of those damaged proteins from the ER back into the cytoplasm, where they are delivered to the proteasome for degradation (7, 16). However, ERAD has been extensively reviewed elsewhere and is not the prime focus of this review (16, 17). Moreover, excessive or sustained ER stress triggers apoptosis to remove the affected cell (18, 19). However, when these mechanisms remain unsuccessful to restore ER homeostasis or recover ER, other stress-response pathways such as autophagy (macroautophagy) may be initiated to selectively eliminate the misfolded/unfolded proteins and damaged ER.

Autophagy is the major lysosomal degradation pathway characterized by the sequestration of the damaged cytoplasmic components by double-membrane bounded vacuoles called autophagosomes (20, 21). This process has been extensively reviewed by others (22, 23) and therefore the fundamental steps in the formation and maturation of autophagosomes are not discussed here in detail. Nevertheless, the association between autophagy and ER stress is not yet fully understood. Therefore, in this review, we have summarized the current findings that integrate the signaling pathways linking autophagy and ER stress.

Each arm of the UPR regulates autophagy in different ways during ER stress. Upon ER-stress, IRE1dissociates from BiP and gets activated (11, 24), which then recruits tumor necrosis factor receptor-associated factor 2 (TRAF2)and apoptosis signal-regulating kinase-1 (ASK1) resulting in the activation of c-Jun N-terminal kinases (JNKs). Active JNK mediates the phosphorylation of Bcl-XL/Bcl-2, leading to the release of Beclin-1 (BECN1) and enhanced basal autophagy (11, 25). PERK arm of UPR also regulates autophagy through the activation of ATF4 and CHOP, which drives the expression of autophagy proteins, autophagy-related (ATG)-12 and ATG5, respectively. ATG12 and ATG5 complex with ATG16L to initiate the formation of autophagosomes (26). CHOP also activates tribbles-homolog3(TRIB3) which inhibits AKT/mechanistic-target-of-rapamycin (mTOR) signaling leading to the induction of autophagy (27). Additionally, calcium released from the ER activates enzymes such as death-associated protein kinase (DAPK), protein-kinase-Cθ (PKCθ) or AMP-activated protein kinase (AMPK), which positively regulate autophagy by inhibiting mTOR (21). However, these signaling pathways have been implicated under different stress conditions, thus they could be functioning in a context-dependent manner.

ER STRESS AND INFLAMMATION OR INFLAMMATORY CYTOKINE REGULATION UNDERLIES ER STRESS

A well-regulated protein homeostasis is crucial for better execution of basic cellular functions. ER plays a significant role

in folding and modifying the secretory as well as membrane proteins, thus maintaining proteostasis (28). Perturbations in protein homeostasis lead to ER stress, which activates UPR and inflammation even in the absence of infection (29). However, ER stress-induced inflammation associated with pathologies such as diabetes, obesity, atherosclerosis, and cancer has been shown to be detrimental. Thus, the destructive or protective nature of ER-stress regulated inflammation depends on the intensity, and type of immune response. Acute induction of ER stress and inflammation safeguard the cell viability and functions while chronic induction can be destructive. Thus, ER stress and ER stress-induced inflammation act in a context-dependent manner. ER stress-induced UPR signaling is coupled with the activation of pro-inflammatory pathways, mediated by nuclear factor kappa light chain enhancer of activated B cells (NF-κB) (4). All the three ER stress sensors; IRE1, PERK and ATF6 induce inflammation by activating NF-kB via different mechanisms leading to the transcription of genes that encode for inflammatory cytokines (29, 30). Under basal conditions, NF-κB forms a complex with inhibitor kappa B (IkB) which prevents the translocation of NF-κB and subsequent transcriptional function. Upon activation of PRR signaling, IκB is phosphorylated and degraded by proteasomes allowing NF-κB to translocate into nucleus and induce the expression of cytokines (31).

IRE1, the evolutionarily conserved signal transducer of the UPR plays a significant role in basic cellular functions and in various pathological conditions associated with inflammation (32). Upon activation of UPR, IRE1 is phosphorylated resulting in the recruitment of TRAF2 and ASK-1 which subsequently activates JNK and NF-κB, leading to the production of inflammatory cytokines. Additionally, IRE1/TRAF2/ASK1 complex activates inhibitory kappa B kinase (IKK), which phosphorylates IκB, allowing the translocation of NF-κB into the nucleus where cytokine gene expression is induced. PERK is also shown to activate NF-κB triggering persistent inflammatory response through the expression of genes that encode inflammatory cytokines like interleukin-1 (IL-1), interleukin-6 (IL-6), and tumor necrosis factor alpha (TNF-α). PERK also regulates NF-κB and apoptosis through the activation of eIF2a-ATF4-CHOP axis of UPR. Activation of PERK downregulates global protein translation, which preferentially affects IκB expression over NF-κB as IκB has a shorter half-life, enabling NF-κB to translocate. ATF6 also positively affects NF-κB activation via mTOR/AKT signaling. Furthermore, reactive oxygen species (ROS) generated because of calcium dysregulation during ER-stress could activate inflammation through NF-κB dependent or independent mechanisms.

Transcription factors such as activator-protein-1 (AP-1) regulated by mitogen activated protein kinases (MAPK) such as p38 work in concert with NF-κB to induce cytokines. Interestingly, IRE1, PERK and ATF6 have been reported to induce inflammation by promoting extracellular-signal-regulated kinase (ERK) and p38. UPR is also known to activate cytokines such as interferon-β (IFN-β) through interferon-responsive-factor-3 (IRF3). Although the precise mechanism remains elusive, stimulator-of-interferon-gene (STING) activation due to calcium disruption induces ER-stress (33, 34). Mitochondrial-DNA released as a result of ER-stress induced mitochondrial damage is also known to activate STING (35). As noted, certain types of ER stress mobilize STING translocation and STING-dependent IFN-I production (33).

In addition to UPR activating inflammatory transcription factors NF-κB and IRF3, UPR arms directly regulate cytokine expression (**Figure 1**). Chromatin immunoprecipitation (ChIP) analyses have revealed the binding of XBP1 to the promoters of the IL-6, TNF-α and IFN-βencoding genes (36, 37). Similarly, ATF4 (38) and CHOP (39) bind to IL-6 and IL-23a promoters, respectively.

ER-stress pathways are also known to directly impact on pattern-recognition-receptors (PRRs). ER stress results in inflammasome activation and IL-1β production possibly resulting in pyroptosis. IRE1 and PERK are also known to upregulate thioredoxin-interacting-protein (TXNIP) (40–42) by abrogating micro-RNA (miR)-17. However, PERK directly increases TXNIP expression through ATF5 (41). TXNIP regulates NLRP3 inflammasome, a multicomponent complex that contains caspase-1 and induces the caspase-1–dependent secretion of the pro-inflammatory cytokines IL-1β and IL-18. TXNIP dependent inflammasome activation occurs on mitochondria resulting in mitochondrial damage and further increase in inflammation. IRE1 is also shown to stimulate nucleotide-binding oligomerization domain-containing protein 1/2 (NOD1/2)-mediated production of inflammatory cytokine IL-6 during *Brucella abortus* infection (43). IRE1a also contributes to the lipid-induced activation of NLR family pyrin domain containing 3 (NLRP3) inflammasome (32). This could be inhibited using tauroursodeoxycholic acid (TUDCA) and the IRE1 kinase inhibitor, KIRA6. Furthermore, IRE1 regulates IL-1β and IL-18 expression through the activation of glycogen-synthase-kinase-3β (GSK3β). ER stress may also enable cells to produce IL-1β in response to TLR4 ligation in a TRIF (TIR domain containing adaptor protein inducing interferon beta)-dependent and caspase 8-dependent, but XBP1 and CHOP independent manner (44). Although PRR and ER-stress can induce inflammation directly, PRR stimulation after ER-stress synergistically induces profound inflammation which has been demonstrated using pharmacologic UPR inducers and XBP1 over-expression (36, 39, 45, 46).

On the other hand, PERK, another ER stress sensor is also shown to activate the downstream signaling pathways leading to the dissociation of NF-κB from IkB and its subsequent translocation into the nucleus. This triggers persistent inflammatory responses by activating the expression of genes that encode inflammatory cytokines like IL-1, IL-6, and TNF-α. Additionally, PERK activates its downstream signaling eIF2a-ATF4-CHOP pathway and NF-κB which initiates inflammation and apoptosis. ERS leads to dissociation of TRAF from TRAF2-procaspase 12 complex, which is located on the ER membrane, leading to activation of caspase 12. The ATF6 pathway also activates NF-κB, further intensifying the expression of inflammatory genes, which secrete more cytokines.

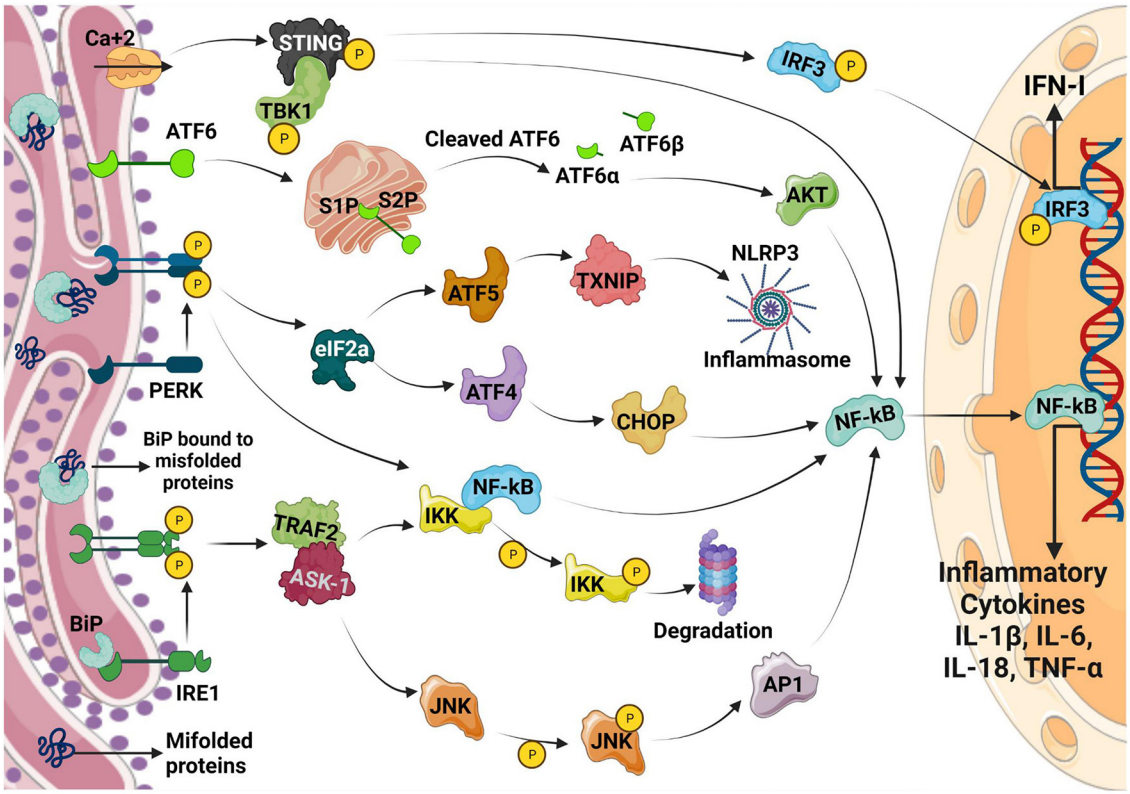

FIGURE 1 | Inflammatory/Cytokine signaling regulated by UPR. Upon ER stress, the ER chaperone BiP dissociates from its complex with IRE1, PERK and ATF6 which results in the activation of the three arms of the UPR pathway as shown in the figure. All the three arms of the UPR regulates the production of inflammatory cytokines via different mechanisms which converge on NF-κB activation. PERK also regulates TXNIP through the induction of ATF5 which in turn modulates NLRP3 inflammasome resulting in enhanced inflammation. Calcium disruption induced ER stress activates STING pathway resulting in the production of IFN-1.

CROSSTALK BETWEEN ER STRESS-AUTOPHAGY-INFLAMMATION IN DISEASE PROGRESSION

PRR induced-inflammation is beneficial in mounting an immune response against microbes. However, profound inflammation in the absence of infection is pathologic which is supported by the clinical use of antibodies against inflammatory cytokines to treat diseases such as intestinal bowel disease, rheumatoid arthritis, and multiple sclerosis.As discussed above, UPR signaling and autophagy are intertwined with inflammation (**Figure 2**). Therefore, UPR has been linked with several inflammatory diseases and some of which has been reviewed here below.

Intestinal Bowel Disease

Intestinal epithelial cells (IECs) are constantly exposed to microbiota, metabolites and toxins which force them to produce large amounts of cytokines and various other proteins resulting in ER-stress. Although, UPR helps in resolving ER-stress, continued ER-stress and disruptions in the UPR mechanism can result in chronic inflammation. Therefore, it is no surprise that studies have associated UPR dysregulation with Crohn's disease (CD) and ulcerativecolitis (UC), two major types of IBDs (47, 48). IBD is also one of the first polygenic disease to be genetically linked to UPR components (47). Intestinal inflammation is primarily linked to IRE1-XBP1 arm of the UPR pathway because mice deleted of the IRE1 gene in mouse intestinal epithelium are more susceptible to dextran sulfate sodium (DSS)-induced colitis (49). Similarly, mice deficient in XBP-1 in the intestine develop spontaneous intestinal inflammation and immune infiltration resembling IBD (50).

The barrier between microbial flora of the gut and IECs is maintained by the secretion of mucin2 (*MUC2*). "Winnie" and "Eeyore"mice engineered with a single nucleotide polymorphism (SNP) expresses misfolded MUC2 resulting in strong UPR induction, and gut inflammation (51, 52). UPR and intestinal inflammation has been also linked in humans. For instance, anterior gradient 2 (AGR2) encoding a protein-disulfide-isomerase which enables protein folding and orosomucoid-like 3 (ORMDL3), which regulates ER calcium have been shown to induce UPR (53–55). Interestingly, $Agr2^{-/-}$ mice develop severe ileo-colitis which is associated with misfolded mucin induced-ER stress (56). Furthermore, genome-wide association studies (GWAS) have mapped the XBP-1 gene locus as an IBD susceptibility region (57, 58). As described before, UPR interacts with autophagy pathways at multiple levels. UPR

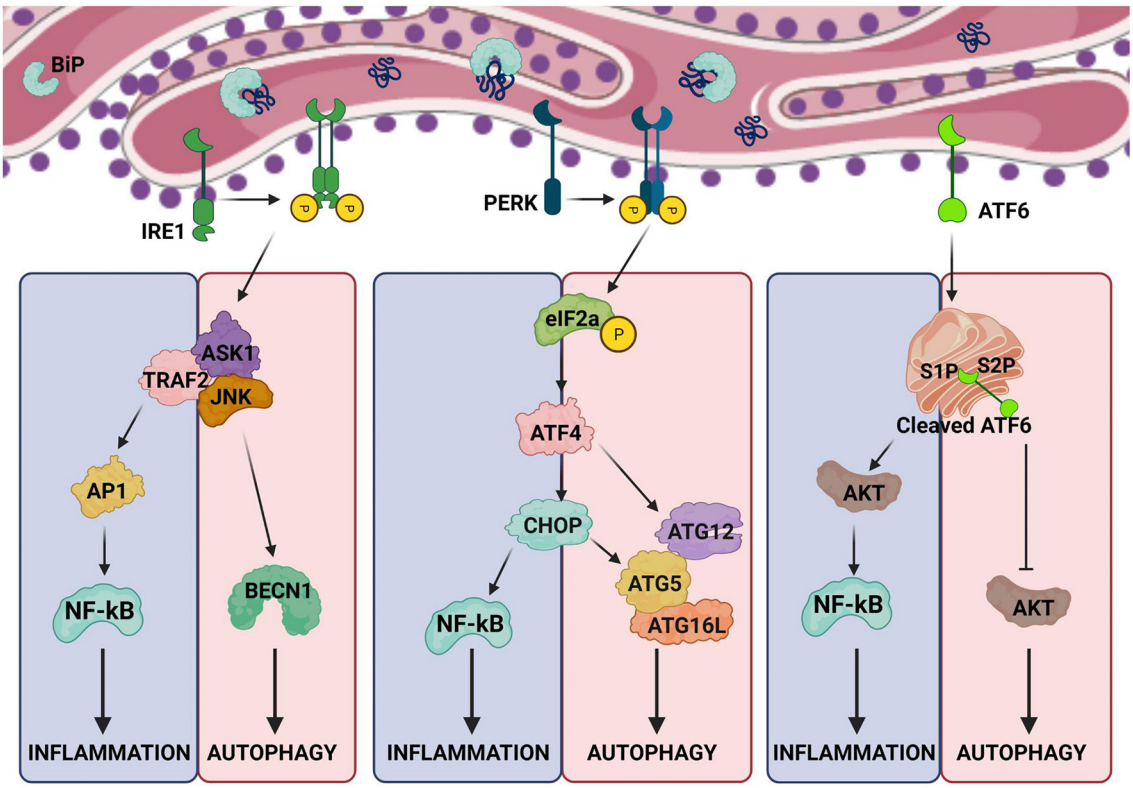

FIGURE 2 | Crosstalk between ER stress, autophagy and inflammation. The arms of UPR activating inflammation also intersect with pathways regulating autophagy. The possible points of intersection are shown in the illustration.

induces autophagy and reciprocally, autophagy may limit UPR by reducing ER-stress (59). Interestingly, a core autophagy effector protein ATG16L is associated with IBD in humans. Consistently, mice deficient in ATG16L1 in IECs develop Crohn's like disease (60–62). Furthermore, deletion of ATG16L1 and XBP1 in IECs results in more severe IBD suggesting that autophagy and UPR synergizes in regulating intestinal inflammation (61).

Chronic Obstructive Pulmonary Disease

UPR is also associated with the pathogenesis of chronic obstructive pulmonary disease (COPD). External stimulants such as cigarette smoke induces ROS production which disturbs the redox environment thus preventing proper protein folding by modulating the protein-disulfide-isomerase (PDI) (63). Dysregulation of protein folding in lung and bronchial epithelial cells induces UPR (64, 65). Furthermore, oxidative damage of proteins caused by cigarette smoke leads to impaired degradation of misfolded, non-functional proteins triggering UPR (66). Cigarette smoke induced-UPR is characterized by PERK-eIF2a-mediated CHOP induction (64, 66–68). On the other hand, cigarette smoke also activates ERK1/2 and NF-kB regulated inflammation (69, 70). Impaired autophagy has been linked to cigarette smoke induced inflammation. On the contrary, activating autophagy using mTOR inhibitor rapamycin results in increased apoptosis and inflammation (71). Interestingly, another form of autophagy known as chaperone-mediated autophagy (CMA), which is LAMP2A facilitated selective degradation of proteins containing Lys-Phe-Gln-Arg-Gln (KFERQ) in the lysosomes mitigates cigarette smoke induced UPR and apoptosis (72).

Neurodegenerative Disorders

Multiple Sclerosis

MS is an autoimmune disorder in which the T-cells target myelin sheath (73). ER-stress induced UPR is found to be a hallmark of MS (74). It is proposed that autophagy-induced cell death could be a possible mechanism by which UPR resolves ER-stress. Hence, autophagy is elevated in MS-lesions resulting in demyelination and neuro-inflammation. PERK and CHOP activation has been found to be consistent with upregulation of BAX and BCL2 in experimental autoimmune encephalomyelitis (EAE). However, the molecular mechanisms integrating UPR, autophagy and inflammation has not been completely understood (3).

Parkinson's Disease

Parkinson's Disease (PD) is a neurodegenerative disease and numerous evidences suggest that inflammation exacerbates the disease (75). Reports have also linked the role of ER-stress in the pathogenesis of PD using neurotoxic models of PD.

Interestingly, depletion of CHOP protects dopaminergic neurons against hydroxydopamine (6-OHDA) indicating the involvement of ER-stress in PD (76). Similarly, silencing XBP1 another UPR arm results in chronic ER stress and dopaminergic neuron degeneration (77). Parkin an E3 ubiquitin ligase implicated in Parkinson's disease is a key regulator of mitochondria-specific autophagy (mitophagy). Interestingly, ATF4 upregulates parkin by directly binding to the promoter region upon ER stress (78). Although, studies addressing the cross talk between ER stress, autophagy and inflammation in PD are limited, UPR can co-regulate inflammation and autophagy as discussed above.

Cardiovascular Diseases

Inflammation and autophagy are known to play a key role in the progression of cardiovascular diseases (CVDs) such as atherosclerosis, ischemia and/or reperfusion. Intriguingly, ER stress that is implicated in inflammation and autophagy has been currently coupled with the pathophysiological aspects of the cardiovascular system (CVS) (79). Upregulation of UPR is observed in cardiac hypertrophy and heart failure. Inflammation and ER stress within the CVS are connected through various regulators such as NF-κB, JNK, spliced XBP-1 and ROS (80–82). As discussed in earlier sections of this review, UPR activation leads to recruitment of TRAF2 by IRE1 which interacts with JNK and IκB resulting in the activation of downstream inflammatory signaling and cytokine production. Additionally, IRE1 auto-phosphorylates and splices its downstream XBP-1 which stimulates the production of inflammatory cytokines by enhancing Toll-like receptor (TLR) signaling (32, 83). ATF6 activation also results in transcriptional activation of inflammatory proteins like C-reactive protein (CRP) which fosters the expression of monocyte chemoattractant protein-1 (MCP-1) and contributes to inflammation (84, 85). Furthermore, ATF6 phosphorylates AKT and activates NF-κB which stimulates the expression of various cytokines (86). Similarly, PERK also triggers NF-κB-induced cytokine signaling by activating IκB (87).

It is well-established that ER stress is also implicated in atherosclerosis where UPR activation is observed in macrophage-derived and smooth muscle cell (SMC)-derived foam cells (79, 88). The plaque deposition in the arterial walls triggers infiltration of macrophages and neutrophils leading to production of IL-1 and IL-6 (89). Additionally, ROS is induced resulting in UPR activation which can further enhance inflammation and tissue damage (89). Inflammation induced mitochondrial damage and ROS can in turn induce autophagy (90). A weak association between autophagy and plaque formation has been reported based on the expression of autophagy markers (91, 92). However, whether autophagy is beneficial or detrimental in atherosclerosis is poorly understood.

CONCLUSION

Traditionally, engagement of PRRs with PAMPs has been considered the primary trigger for inflammation. However, changes in intracellular functions causing cellular stress have been lately recognized to play a key role in inflammation associated with pathologies. Thus, ER stress-induced inflammation has been implicated in several inflammatory diseases. Although response to ER-stress (UPR) aids in mitigating ER-stress, UPR pathways also promote inflammation and diseases such as diabetes, obesity, IBD, inflammatory lung disorders, cardiovascular diseases and cancer. Moreover, UPR pathways are interlinked with other cellular-stress response mechanisms such as autophagy which can potentially mitigate inflammation and disease progression. Conversely, activation of cellular homeostasis mechanisms such as autophagy can be an impediment to treat diseases such as cancer. However, UPR induced inflammation and autophagy vary between diseases and is cell type dependent. Although, inflammation and autophagy have been reported during ER-stress, it is correlative. Therefore, molecular mechanisms that integrate UPR, autophagy and inflammation need to be elucidated which is crucial for therapeutic targeting.

AUTHOR CONTRIBUTIONS

SC: prepared the manuscript draft and figures. All authors contributed to the article and approved the submitted version.

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mmu-miR-145a-5p Accelerates Diabetic Wound Healing by Promoting Macrophage Polarization Toward the M2 Phenotype

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Diabetic wounds are recalcitrant to healing. One of the important characteristics of diabetic trauma is impaired macrophage polarization with an excessive inflammatory response. Many studies have described the important regulatory roles of microRNAs (miRNAs) in macrophage differentiation and polarization. However, the differentially expressed miRNAs involved in wound healing and their effects on diabetic wounds remain to be further explored. In this study, we first identified differentially expressed miRNAs in the inflammation, tissue formation and reconstruction phases in wound healing using Illumina sequencing and RT-qPCR techniques. Thereafter, the expression of *musculus* (mmu)-miR-145a-5p ("miR-145a-5p" for short) in excisional wounds of diabetic mice was identified. Finally, expression of miR-145a-5p was measured to determine its effects on macrophage polarization in murine RAW 264.7 macrophage cells and wound healing in diabetic mice. We identified differentially expressed miRNAs at different stages of wound healing, ten of which were further confirmed by RT-qPCR. Expression of miR-145a-5p in diabetic wounds was downregulated during the tissue formation stage. Furthermore, we observed that miR-145a-5p blocked M1 macrophage polarization while promoting M2 phenotype activation *in vitro*. Administration of miR-145a-5p mimics during initiation of the repair phase significantly accelerated wound healing in db/db diabetic mice. In conclusion, our findings suggest that rectifying macrophage function using miR-145a-5p overexpression accelerates diabetic chronic wound healing.

Keywords: mmu-miR-145a-5p, diabetes, wound healing, macrophage, inflammation

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INTRODUCTION

Wound healing is a complex process involving a variety of cell types and the coordination of various cytokines to regulate wound repair. Macrophages tightly participate in and regulate the entire process of wound healing, and their numbers increase during the inflammation phase, peak during the tissue formation phase, and gradually decline during the maturation phase (1, 2). Generally, macrophages are divided into M1 (classical activation) and M2 (alternative activation) phenotypes. M1 macrophages phagocytize pathogens and necrotic tissues and secrete inflammatory factors, while M2 macrophages help to relieve inflammation and promote angiogenesis by releasing anti-inflammatory factors and growth factors. Diabetic patients experience wounds characterized by

a prolonged inflammatory state, with overactivated M1-like macrophages and elevated levels of proinflammatory factors with inadequately activated M2 phenotypes and impaired expression of growth factors (3, 4). Because macrophages are an important target for the treatment of chronic diabetic wounds, studying the regulation of macrophage polarization may uncover therapeutic avenues to accelerate wound repair in diabetes.

miRNAs, 20–24 nucleotide (nt) non-coding RNAs, have the ability to regulate gene expression and the network of cellular processes. One miRNA can regulate the expression of multiple target genes, while genes involved in the same physiological or pathological process may be regulated by a single or a few miRNAs. Accordingly, miRNAs have advantages over conventional genes when used as therapeutic targets in diseases (5). Many studies have determined that miRNAs are important in regulating macrophage polarization (6, 7). Furthermore, M1 and M2 phenotypes play distinctive roles in the progression of refractory diabetic wounds. Thus, miRNAs might affect the repair of diabetic wounds by regulating the conversion between M1 and M2 macrophages.

In the present study, we determined the miRNA expression profiles in different stages of wound healing using Illumina sequencing and real-time quantitative reverse transcript polymerase chain reaction (RT-qPCR) techniques. Furthermore, overexpression of mmu-miR-145a-5p using mimics inhibited M1 polarization and promoted M2 polarization *in vitro*. Administration of miR-145a-5p mimics during the initiation of the repair phase also significantly accelerated wound healing in diabetic mice.

MATERIALS AND METHODS

Animals

BKS-Lepr^{em2Cd479}/Nju (db/db) diabetic mice (male, 8–10-week-old) were purchased from the Peking University Experimental Animal Center (Beijing, China). The measured blood glucose value of the db/db mice was 23.86 ± 2.07 mmol/L while the normal was ∼6 mmol/L, indicating that the diabetic mouse models were successfully established. Normal C57BL/6J mice (male, 8–10-week-old) were purchased from Weitonglihua Co., Ltd. (Beijing, China). All animals were maintained in an animal facility, where the temperature was 22 ± 2°C and the humidity 55 ± 10% on a 12 h light-dark cycle. Food and water were freely available.

Wound Healing Model

Mice were anesthetized with pentobarbital (i.p., 80 mg/kg). The dorsal surface was then shaved and cleaned with 75% ethanol. Two round wounds with a diameter of 5 mm were made on each side of the midline of the shaved dorsum using a punch biopsy tool (Miltex, USA). The wounds were left undressed, and all mice were housed separately thereafter. The wounds were imaged using a high-resolution camera, the sizes of which were calculated using ImageJ software (National Institutes of Health, USA). Biopsies were harvested on postoperative days 0, 1, 3, 5, 7, 15, 19, and 21.

RNA Isolation and Library Preparation

During the inflammation, tissue formation, and tissue reconstruction phases of skin wound healing in control mice, namely, at 0, 1, 3, and 7 days post-operation, the wounds and a 5 mm unwounded skin border were harvested and stored at −80°C until further processing. Total RNA was isolated using the miRNeasy Serum/Plasma Kit (Qiagen, USA). The A (260/280) absorbance ratio of isolated RNA was 1.8–2.0, while the A (260/230) absorbance ratio was >1.6. Integrity of total RNA was determined using formaldehyde denaturing gel electrophoresis. The libraries were constructed from total RNA using the Illumina TruSeq Small RNA Sample Preparation kit (Illumina, USA). Single-stranded cDNA was created by reverse transcription reactions with the ligation products. The cDNA was amplified by PCR. Finally, Illumina sequencing technology was used to sequence these prepared samples.

Illumina Sequencing and RT-QPCR Validation

Raw sequences were processed using the Illumina pipeline program. Clean reads were filtered, and contaminated reads were removed. Secondary structure prediction of individual miRNAs was performed using Mfold software. Clean sequence reads were mapped using miRBase 20.0, and a mismatch of 1–2 nucleotide bases was allowed. All data were transformed to log base 2. Differences between the samples were analyzed using chi-square and Fisher's exact tests. miRNAs with a fold difference N2.0 and $P < 0.05$ were considered statistically significant. miRNAs with high expression abundance and significant differences (fold change >4) in each phase of skin trauma were verified using RT-qPCR with Applied Biosystems TaqMan^{TM} MGB (minor groove binder) probes (Thermo Fisher Scientific, Waltham, MA, USA). The experiment was performed in strict accordance with the reagent instructions. All samples were analyzed in triplicate. The specificity of each PCR product was validated by melting curve analysis at the end of PCR cycles, and the cycle threshold (Ct) value was defined as the number of cycles required for the fluorescent signal to reach the threshold. Relative expression levels of miRNAs in wound samples were calculated using the $2^{-\Delta\Delta Ct}$ formula, where $\Delta\Delta Ct$ = [Ct (target, test) – Ct (ref, test)] – [Ct (target, calibrator) – Ct (ref, calibrator)]. All primers used were obtained from Invitrogen (Carlsbad, CA, USA).

Transfection of miR-145a-5p Mimics and Negative Control

RAW 264.7 macrophage cells were grown in Roswell Park Memorial Institute medium (RPMI) 1640 (Gibco, USA) containing 10% fetal bovine serum (FBS, Gibco), penicillin (100 U/mL), and streptomycin (100 μg/mL) at 37°C in a 5% CO_2 atmosphere. Macrophage polarization was induced with 1 μg/ml lipopolysaccharide (LPS, Sigma, L2880) or 20 ng/ml interleukin-4 (IL-4, Proteintech, 214-14). The cells were then transfected with 4 nM miR-145a-5p mimics (4464066, MC11480, Thermo Fisher Scientific) and negative control (4464059, Thermo Fisher Scientific) using Lipofectamine RNAiMAX (Invitrogen)

according to the instructions and were harvested 48 h later. Three repeats were performed for each group.

For the *in vivo* study, miR-145a-5p mimics and negative control were diluted to 4 nM in autoclaved phosphate-buffered saline (PBS) and injected subcutaneously around the wound at a volume of 4 μL in each quadrant. The injections were repeated every 3 days for 2 weeks.

Quantitative Real-Time PCR

At predetermined time points, total RNA was isolated from skin tissues and RAW 264.7 macrophage cells using TRIzol reagent (Invitrogen). Thereafter, complementary DNAs (cDNAs) were synthesized using the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. The resulting cDNAs were amplified with 40 cycles by RT-qPCR using Power SYBR@ Green PCR Master Mix (Thermo Fisher Scientific). Each sample was analyzed three times and normalized to β-actin as the internal control. Primer sequences for RT-qPCR are listed in **Table 1**.

Histological Analysis and Immunohistochemistry

The wounds and a 5 mm unwounded skin border were harvested from mice and processed for histological analysis. Wound widths were measured as the distance between wound margins. Wound areas were determined using image analysis (measured below the clot and above the panniculus muscle). Re-epithelialization was defined as the percentage of distance migrated by the neoepidermis compared to the upper wound width. Immunohistochemistry analysis was performed on formalin-fixed, paraffin-embedded 5-μm sections. The primary antibodies used in this study were against keratin 14 (K14, 1:1000, Covance, Princeton, NJ, USA), Ki67 (1:1000, Covance) and CD206 (1:500, Novus Biologicals, CO, USA). To quantify CD206 expression, ImageJ 1.50d (USA) was used to analyze the integrated optical density.

Bioinformatics Analysis of Downstream Target Genes of mmu-miR-145a-5p

To predict target genes of mmu-miR-145a-5p, TargetScan (5.0), miRanda (3.3a), and PITA (1.0) analyses were conducted and the default thresholds were targetscan_score $\geq$50, miranda_Energy < -10, and ddG <-5. Thereafter, the intersections of the three databases were selected as candidate downstream miRNAs of mmu-miR-145a-5p for further analysis. Functional enrichment analysis of the target genes of miR-145a-5p was conducted using clusterProfiler with Gene Ontology.db. (GO) and the Kyoto Encyclopedia of Genes and Genomes.db. (KEGG). Significance was assessed using hypergeometric tests and Benjamini-Hochberg correction, with a significance threshold of $p < 0.05$ (8, 9).

Statistical Analysis

All results are presented as the mean ± SD. All experiments were performed with a minimum of three independent replicates. One-way ANOVA followed by Bonferroni *post-hoc* tests were performed to analyze multiple groups and unpaired Student's *t*-test was performed to analyze two parallel groups, using SPSS software (IBM, Armonk, NY, USA). Differences were considered significant at two-sided $p < 0.05$.

RESULTS

Differentially Expressed miRNAs During Wound Healing in Mice

High-throughput miRNA sequencing (miRNA-Seq, 10 million reads/sample) was performed on unharmed skin tissues (D0) and during inflammation (postinjury day 1, D1), tissue formation (postinjury day 3, D3) and tissue reconstruction (postinjury day 7, D7) phases during wound healing in normal male C57BL/6J mice. **Table 2** shows the total number of differentially expressed miRNAs between different groups (fold change $\geq$2 and $P \leq 0.01$). The miRNAs with high expression abundance and significant differences (a fold change >4) in each phase of skin trauma were verified using real-time PCR with the probe method. The verification results for 10 miRNAs were consistent with the sequencing results, and expression levels of mmu-miR-183a-5p, mmu-miR-143-3p, mmu-miR-145a-5p, mmu-miR-let-7c, mmu-miR-26a-5p, mmu-miR-27a-3p, mmu-miR-30e-5p, mmu-miR-1a-3p, mmu-miR-1b-5p, and mmu-miR-let-7b were significantly downregulated 1–7 days after trauma (**Figure 1**). These results suggest that miRNAs participate in the regulation of different stages of wound healing and might be potential therapeutic targets in diseases related to abnormal wound repair.

An Excessive Inflammatory Response and Insufficient Growth Factor Secretion During the Formation Phase of Skin Trauma Causes Delayed Wound Healing in Diabetic Mice

Next, we examined the changes in wound repair and the inflammatory state in diabetic mice. The wound closure speed in the db/db mice was dramatically lower than that in the control group (**Figure 2A**). We also determined cytokine expression in wound tissues and found that expression levels of the proinflammatory cytokines tumor necrosis factor alpha (TNF-α) and interleukin-1alpha (IL-1α) in db/db mice were downregulated on days 1 and 3 and elevated on day 7 post-injury, suggesting insufficient activation of M1 macrophages during the inflammatory stage of the wounds but overreaction during the tissue formation stage (**Figures 2B,C**). Quite a number of studies have reported the abnormal inflammation in chronic diabetic wound, which is characterized by the upregulation of pro-inflammatory cytokines, such as TNF-a, IL-1α, IL-1β, and IL-6 (10–13). Shang et al. found the increased M1-like macrophages on d7 in diabetic mice, which were believed to be closely related to the expression of pro-inflammatory factors (14). In addition, the growth factors transforming growth factor beta 2 (TGF-β2) and Smad5 in the db/db mice decreased significantly at different time points post-injury, indicating reduced polarization of M2 macrophages in all stages of diabetic wound healing (**Figures 2D,E**). Collectively, the failure of transformation from

TABLE 1 | Primers for RT-qPCR analysis in mice.

Genes	Forward primer (5′-3′)	Reverse primer (5′-3′)
TNF-α	CAGGCGGTGCCTATGTCTC	CGATCACCCCGAAGTTCAGTAG
IL-1α	CGAAGACTACAGTTCTGCCATT	GACGTTTCAGAGGTTCTCAGAG
TGF-β2	TCGACATGGATCAGTTTATGCG	CCCTGGTACTGTTGTAGATGGA
Smad5	GAGCCATCACGAGCTAAAACC	ACTGGAGGTAAGACTGGACTCT
CD86	GGTGGCCTTTTTGACACTCTC	TGAGGTAGAGGTAGGAGGATCTT
CD206	CTCTGTTCAGCTATTGGACGC	CGGAATTTCTGGGATTCAGCTTC
Arg-1	TGACTGAAGTAGACAAGCTGGGGAT	CGACATCAAAGCTCAGGTGAATCGG
β-Actin	GGCTGTATTCCCCTCCATCG	CCAGTTGGTAACAATGCCATGT

TABLE 2 | Total number of differentially expressed miRNAs (fold change ≥2 and $P \leq 0.01$).

DEG sample	Total miRNAs	Down regulated miR NO.	Up regulated miR NO.	Total DEmiR
D1 vs. D0	1141	48	60	108 (9.47%)
D3 vs. D0		118	97	215 (18.84%)
D7 vs. D0		110	98	208 (18.23%)
D3 vs. D1		107	66	173 (15.16%)
D7 vs. D1		100	97	197 (17.27%)
D7 vs. D3		25	46	71 (6.22%)

M1 macrophages to M2 phenotypes during the tissue formation stage might be an important mechanism of delayed wound healing in diabetes mellitus.

miR-145a-5p Promotes Macrophage Polarization Toward M2 *in vitro*

miR-145a-5p was one of the differentially expressed miRNAs during wound healing identified by microarray and RT-qPCR. Many studies have reported the regulatory effects of miR-145a-5p on cell differentiation, such as myoblasts, adipocytes, and microglia (15–17). Whether miR-145a-5p affects the polarization of macrophages in diabetic wound healing greatly excited our interest. First, we studied the expression of miR-145a-5p in wound tissues of db/db mice and found that expression levels of miR-145a-5p increased on days 0, 1, and 15 but were significantly decreased on day 7 after the operation (**Figure 3A**). miR-145a-5p was involved in the regulation of inflammation (18), and the higher expression level of miR-145a-5p in db/db mice than in control mice on day 0 might be explained by diabetic microangiopathy and inflammation. These results suggest that miR-145a-5p is involved in the pathophysiological process of diabetic wound healing.

To further clarify the effects of miR-145a-5p on macrophage polarization, we transfected miR-145a mimics or the related negative control (NC) in RAW 264.7 macrophage cells pretreated with LPS or IL-4. Transfection of miR-145a-5p mimics into RAW 264.7 cells increased the expression levels of miR-145a-5p > 1000-fold compared to that of the NC group (**Figure 3B**). Generally, CD86 and tumor necrotic factor-α (TNF-α) are used as biomarkers generated by M1 macrophages, while CD206 and arginase 1 (Arg-1) are accepted markers of the M2 phenotype (19, 20). miR-145a-5p mimic transfection dramatically downregulated expression of CD86 and TNF-α in RAW 264.7 macrophages pretreated with LPS, suggesting that miR-145a-5p abolished M1 polarization induced by LPS (**Figures 3C,D**). On the other hand, miR-145a-5p overexpression caused increased expression of CD206 and Arg-1 in macrophages pretreated with LPS or IL-4, indicating the roles played by miR-145a-5p in facilitating M2 polarization (**Figures 3E,F**). These results demonstrate that miR-145a-5p promotes macrophage polarization toward M2.

miR-145a-5p Mimics Accelerate Wound Healing in db/db Mice

miR-145a-5p mimics were administered locally to assess their therapeutic effect on diabetic trauma. The wound closure rate in db/db mice treated with miR-145a-5p mimics was dramatically higher than that in the control group (**Figures 4A,B**). Histological analysis revealed that wounds in the miR-145a-5p mimic group healed at a faster rate than those in the control group (**Figure 5A**). Immunohistochemistry of K14 showed that the neoepithelial lengths were longer in the healing skin of db/db mice transfected with miR-145a-5p mimics than in those treated with negative control miRNAs, and enhanced expression of Ki67 on postoperative day 7 suggested an increased number of proliferative cells during the tissue formation stage when miR-145a-5p was overexpressed (**Figures 5B,C**). Importantly, miR-145a-5p mimics increased the expression of CD206 in diabetic wounds, suggesting the ability of miR-145a-5p to promote M2 polarization *in vivo* (**Figures 5D,E**). These results show that overexpression of miR-145a-5p accelerates diabetic wound healing and increases M2 phenotypes in diabetic wounds.

Go and KEGG Functional Enrichment Analysis of miR-145a-5p Downstream Genes

In the present study, we screened the downstream genes of miR-145a-5p using 3 databases, including TargetScan, miRanda,

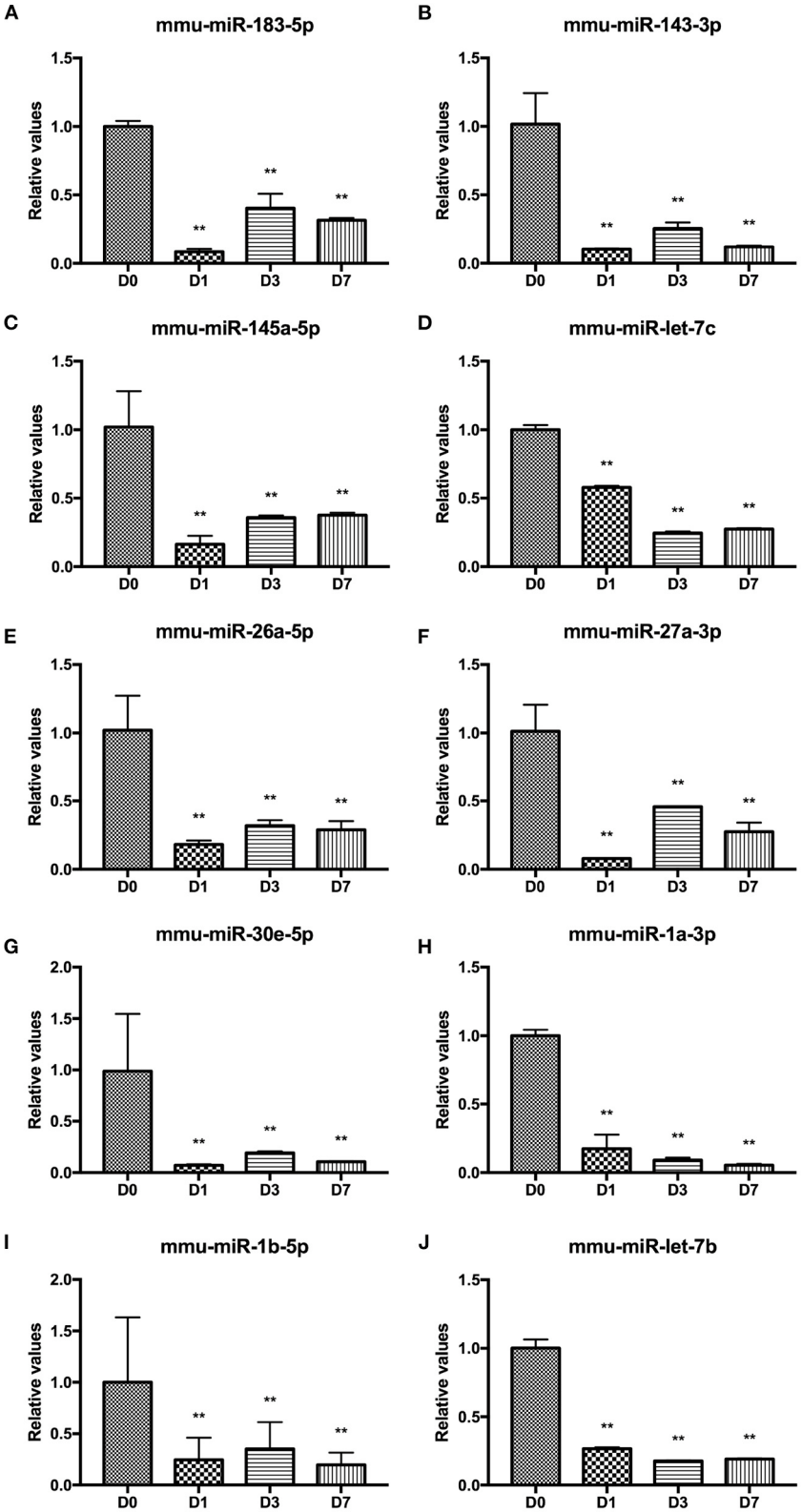

FIGURE 1 | Differentially expressed miRNAs at different stages of wound healing. **(A)** mmu-miR-183a-5p. **(B)** mmu-miR-143-3p, **(C)** mmu-miR-145a-5p. **(D)** mmu-miR-let-7c. **(E)** mmu-miR-26a-5p. **(F)** mmu-miR-27a-3p. **(G)** mmu-miR-30e-5p. **(H)** mmu-miR-1a-3p. **(I)** mmu-miR-1b-5p. **(J)** mmu-miR-let-7b. $^{**}P < 0.01$ vs. control (D0).

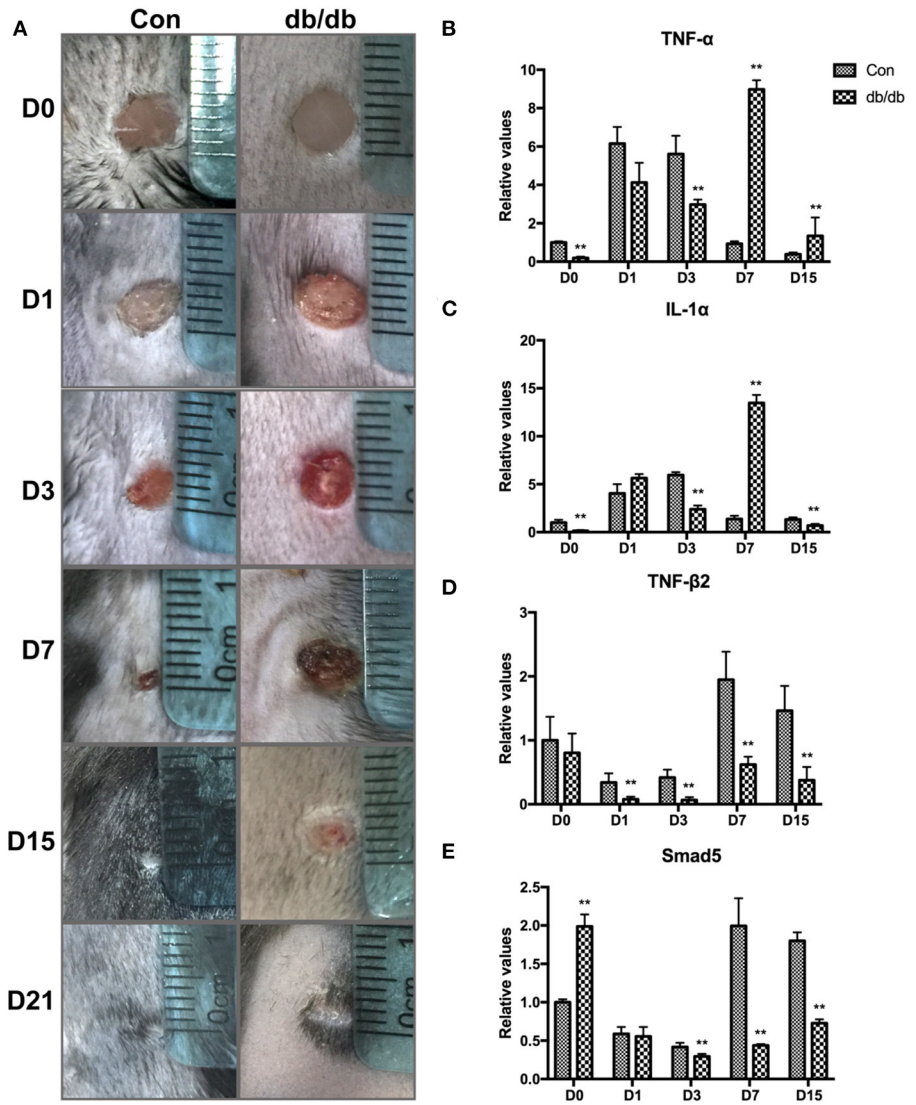

FIGURE 2 | Changes in wound size and the expression of proinflammatory and growth factors in diabetic wound healing. **(A)** Representative images of excisional wounds in db/db and control mice on days 0, 1, 3, 7, 15, and 21. **(B–E)** Expression of proinflammatory and growth cytokines in the db/db and control mice on days 0, 1, 3, 7, and 15. $^{**}P < 0.01$ vs. the corresponding control (Con) group at the same time points.

and PITA. Subsequently, 2,473 target genes of miR-145a-5p predicted by all three databases were selected. GO and KEGG functional enrichment analyses revealed that miR-145a-5p is involved in regulating many biological processes, including signal transduction, cell differentiation, apoptotic processes, etc. (**Figure 6**).

DISCUSSION

The normal wound healing response is characterized by progression from clot formation to an inflammatory phase, a repair phase, and finally remodeling. Macrophages have been verified to be important in the inflammatory phase of tissue repair, where their dynamic plasticity allows these cells to mediate both tissue-destructive and tissue-reparative functions (10). In diabetic chronic wounds, an extended inflammatory phase stemming from macrophage functional disturbance halts this progression (21, 22). The phagocytosis rate of M1 macrophages in the diabetic state is >5 times slower than normal, which may lead to slower clearance of wound necrotic tissue and apoptotic cells and sustained release of inflammatory factors. The blocked transformation from the M1 to M2 phenotype and the insufficient secretion of anti-inflammatory factors and growth factors lead to failure of normal tissue formation and eventually prolonged healing in diabetic mellitus (23, 24). Some studies have attempted to treat diabetic trauma by regulating the macrophage-mediated inflammatory response and have achieved positive effects, suggesting macrophages as a potential

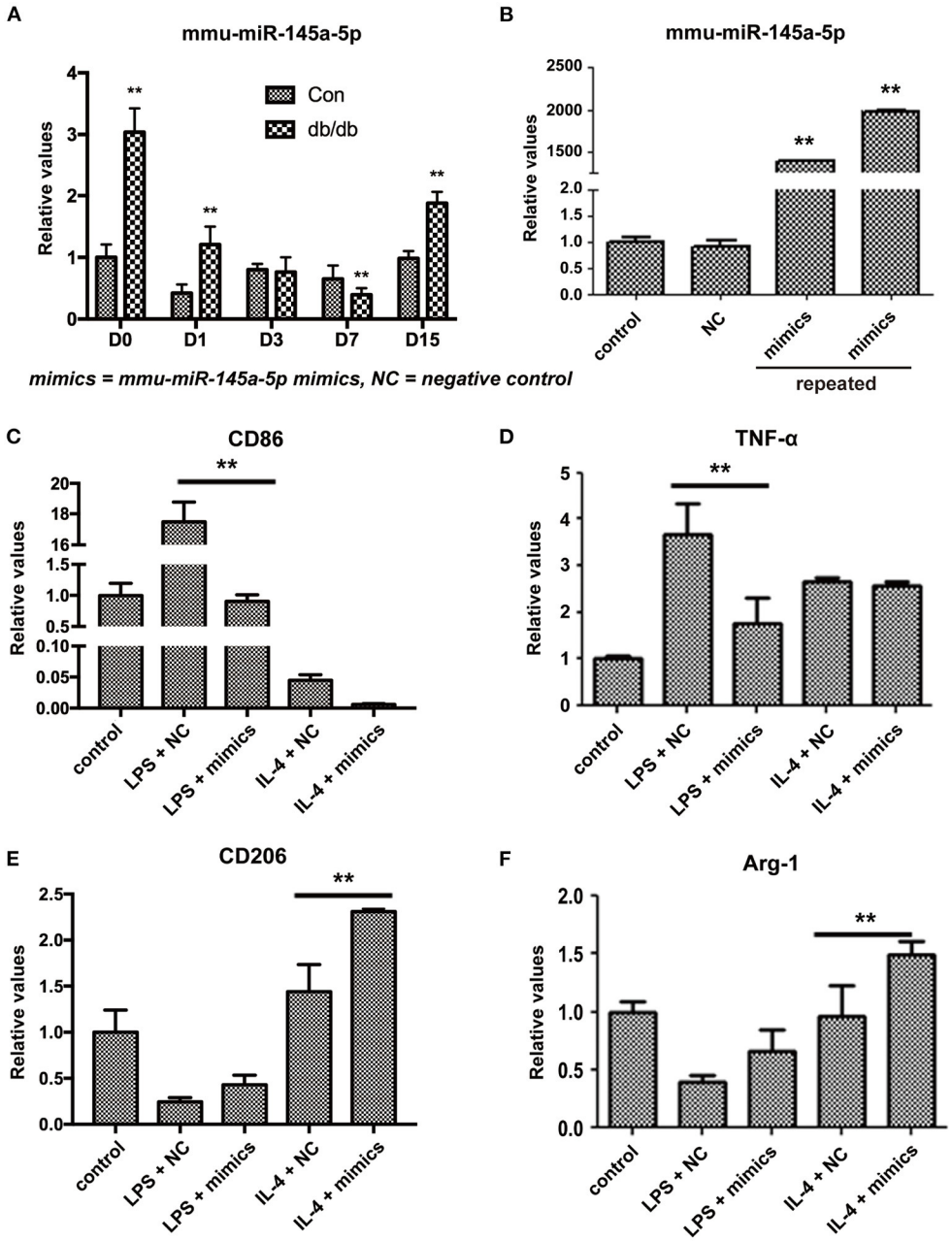

FIGURE 3 | Influence of miR-145a-5p on macrophage polarization in RAW 264.7 cells. **(A)** Expression of miR-145a-5p in db/db and control mice on postoperative days 0, 1, 3, 7, and 15. **(B)** Expression of miR-145a-5p in RAW 264.7 macrophages 24 h after the addition of miR-145a-5p mimics or negative control miRNA. **(C–F)** Expression of CD86, TNF-α, CD206 and Arg-1 in RAW 264.7 macrophages pretreated with LPS or IL-4 in combination with miR-145a-5p mimics/negative control miRNA. $^{**}P < 0.01$ vs. the corresponding control group.

therapeutic target in refractory wounds in diabetic mellitus (25–30). Our research group has been committed to investigating wound healing mechanisms and therapy and previously found that knockdown of casein kinase 2-interacting protein 1 (CKIP-1) in macrophages inhibited M1-type activation and slowed the wound healing rate (31). However, innovative methods still need to be explored to regulate the macrophage response during wound healing. In this study, we identified miR-145a-5p as one of the differentially expressed miRNAs between db/db diabetic mice and controls. miR-145a-5p has been demonstrated to play important regulatory roles in cell differentiation and polarization, such as myoblasts, adipocytes, and microglia (15–17). Therefore, the regulation of miR-145a-5p on the macrophage response and its effect on diabetic wound healing aroused our interest.

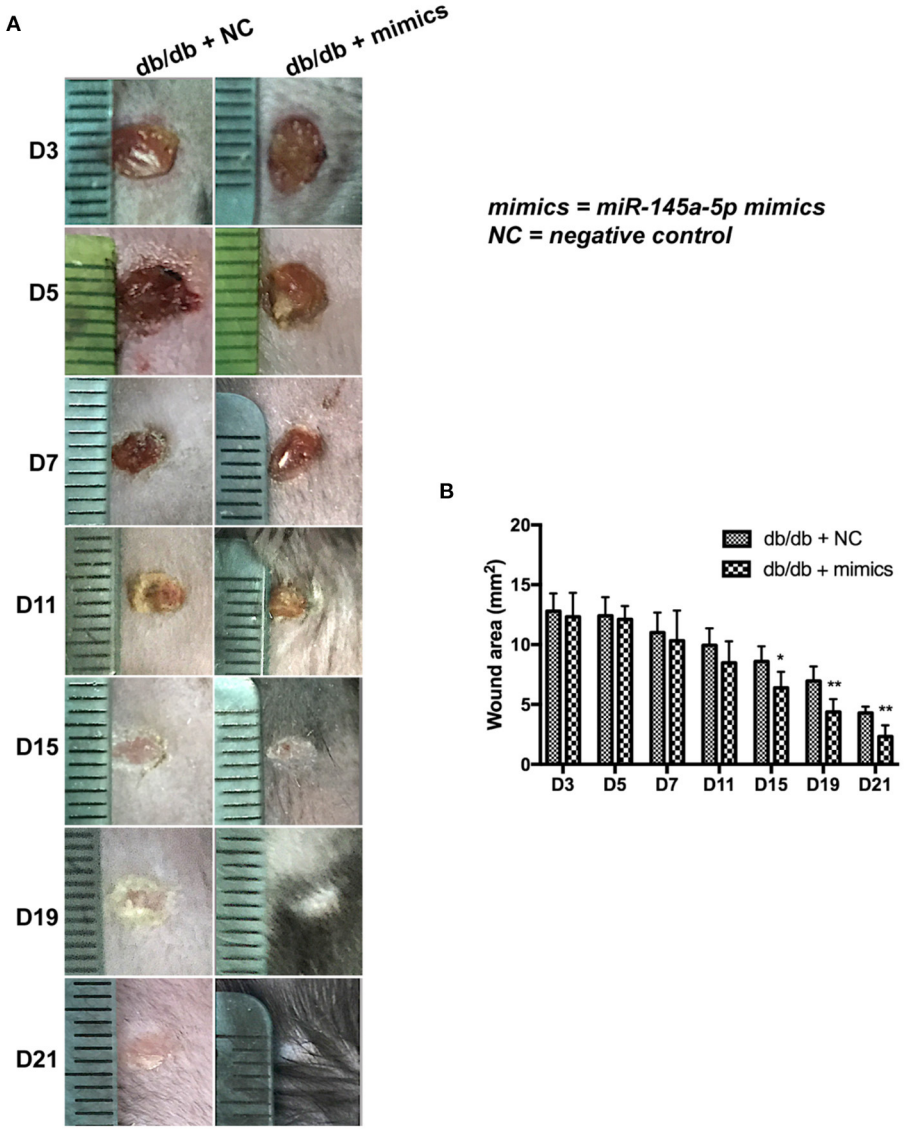

FIGURE 4 | Effect of miR-145a-5p on wound healing size in diabetic mice. **(A)** Representative images of excisional wounds of db/db mice treated with miR-145a-5p mimics or the corresponding negative control. **(B)** Wound size measurement. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. the corresponding control group.

miRNAs are a class of endogenous RNAs of ∼22 nucleotides in length produced by dicer 1, ribonuclease III (Dicer) processing single-stranded RNA precursors of ∼70–90 bases (32). miRNAs bind to the corresponding 3′ untranslated region (3′UTR) of the target mRNA through complete or incomplete pairing, preventing its translation or disrupting its stability to regulate gene expression (33). miRNAs regulate 30% of all human genes and have extensive regulatory functions in life activities and profound effects on gene expression, growth, and behavior (34, 35). miRNAs are widely involved in various skin wound healing processes (36). miR-125b regulates leukocyte aggregation and activation by inhibiting TNF-α expression (37). miR-125b inhibits keratinocyte proliferation by downregulating fibroblast growth factor receptor 2 (FGFR2) (38). In ischemic trauma, hypoxia in the wound site and high miR-210 expression reduce keratinocyte proliferation by inhibiting E2F transcription factor 3 (E2F3) expression (39). miR-483-3p depresses keratinocyte proliferation and migration by downregulating mitogen-activated protein kinase-activated protein kinase 2 (MAPKAPK2, or MK2), Ki67, and Yes-associated protein 1 (YAP1) (40). miR-203 regulates keratinocyte proliferation and migration at the wound margin by affecting RAN and Ras association (RalGDS/AF-6) and pleckstrin homology domain 1 (RAPH1) (41). miR-205 promotes keratinocyte migration by inhibiting inositol polyphosphate phosphatase-like 1 (SHIP2) (42, 43). miR-21 downregulates tissue metalloproteinase inhibitor 3 (TIMP3) and T-cell lymphoma invasion and metastasis 1 (TIAM1) to promote keratinocyte migration (44). miR-21 is elevated in

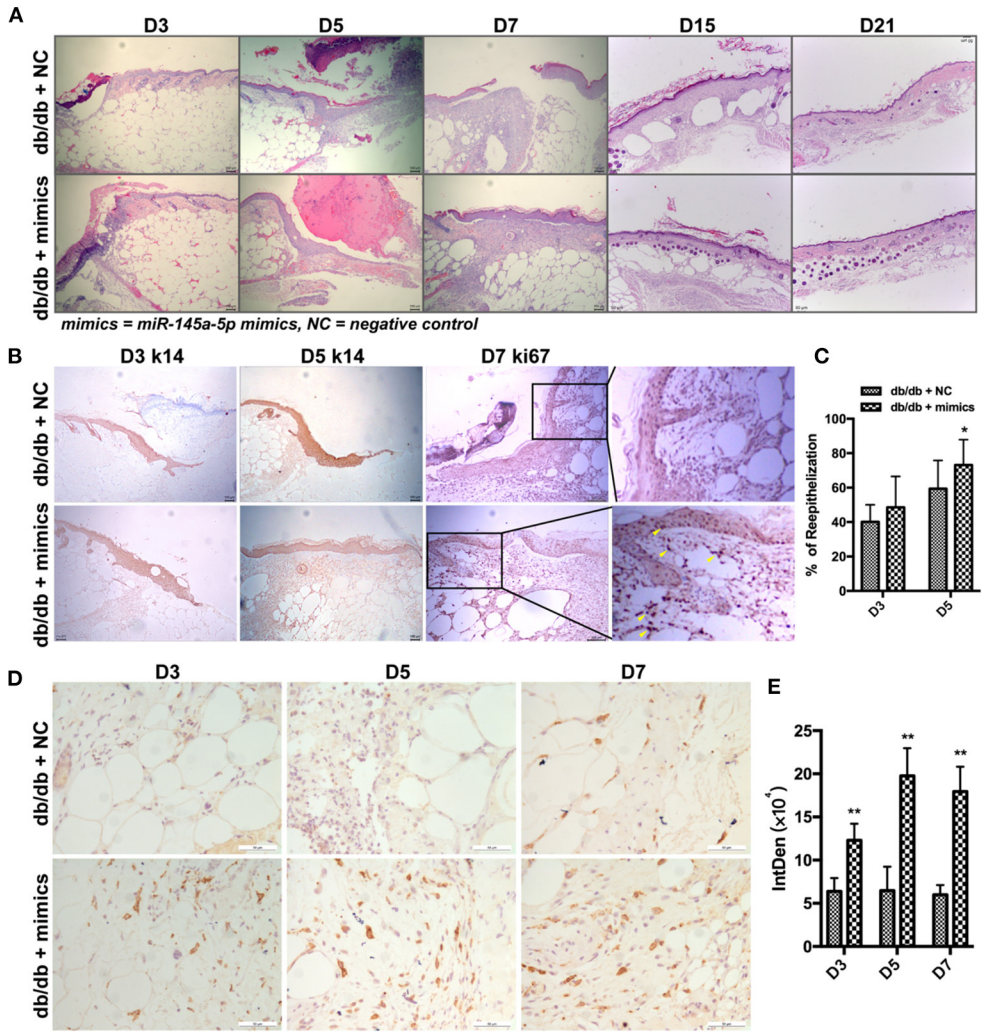

FIGURE 5 | Effect of miR-145a-5p on keratinocyte reepithelialization of wound healing in diabetic mice. **(A)** Representative H&E stained images of excisional wounds in db/db mice treated with miR-145a-5p mimics or negative control on days 3, 5, 7, 15, and 21. **(B)** Representative immunohistochemistry (IHC) stained images of K14 and Ki67 in wounds of db/db mice treated with miR-145a-5p mimics or negative control on days 3, 5, and 7. $Ki67^{+}$ cells are indicated with yellow arrows. **(C)** Statistical analysis of epithelialization ratio. **(D)** Representative IHC images of CD206 in diabetic wounds treated with miR-145a-5p mimics or negative control on days 3, 5, and 7. **(E)** Statistical analysis of the integrated optical density of CD206 from D. $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. the corresponding control group at the same time points.

the skin of diabetic mice and regulates wound contraction and collagen deposition (45, 46). miR-27b inhibits mouse mesenchymal stem cell migration to burn sites by silencing C-X-C motif chemokine ligand 12 (SDF-1A), which slows wound healing (47). Members of the miR-199 family are involved in the regulation of wound healing through the AKT/mammalian target of rapamycin (mTOR) pathway (48). let-7c overexpression inhibits the M1 macrophage phenotype and promotes the M2 phenotype (49). LPS-pretreated mesenchymal cells secrete let-7b from exosomes, promoting macrophage secretion of anti-inflammatory factors and targeting M2 differentiation, while miR-130b inhibits M2 macrophage activation by targeting peroxisome proliferator-activated receptor gamma (PPARγ) (50). In conclusion, miRNAs are intimately involved in the regulation of wound healing. In this study, we screened out miRNAs related to the traumatic inflammatory response, tissue formation, and tissue remodeling by chip and finally verified 10 differentially expressed miRNAs (high expression and significant difference) using RT-qPCR. Among these miRNAs, miR-145a-5p was selected for further study due to its potential to regulate macrophage polarization.

In this study, we found that local application of miR-145a-5p dramatically promoted wound healing in db/db mice. K14 is an intermediate filament protein that is mainly expressed in epithelial cells, which are generally used as a research biomarker of epidermal skin (51). Ki67 is universally expressed among proliferating cells, but is absent from quiescent cells (52). miR-145a-5p was found to facilitate diabetic wound

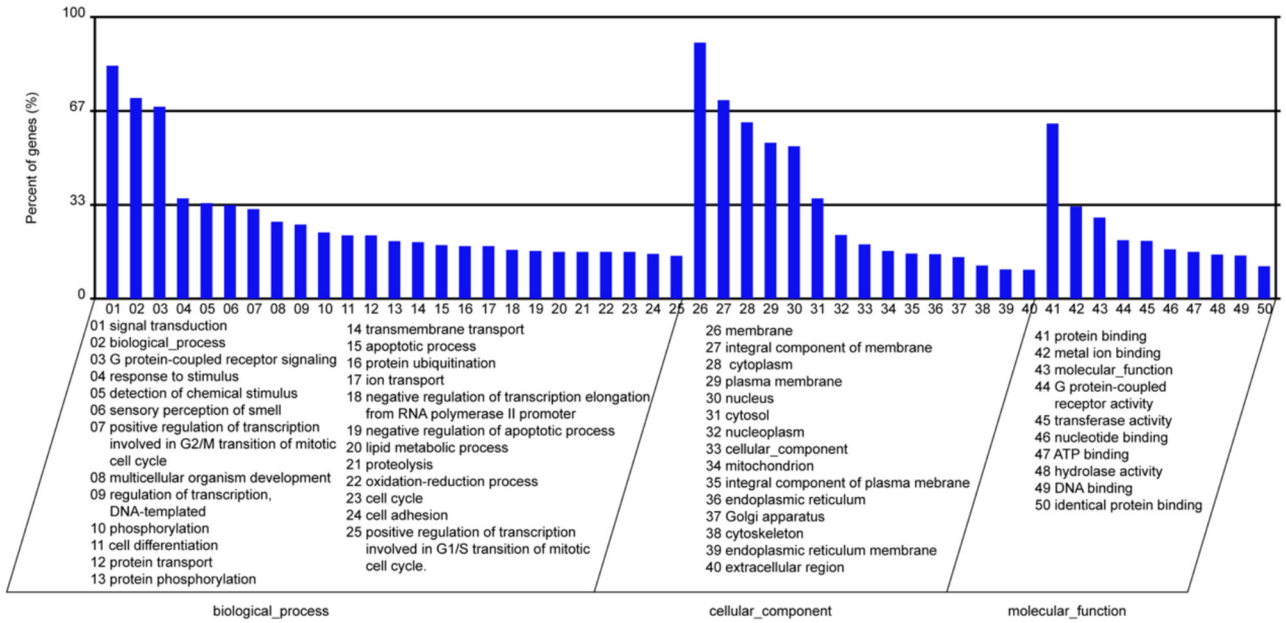

FIGURE 6 | Functional enrichment analysis of the predicted downstream genes of miR-145a-5p.

healing, of which the indicators were chiefly the accelerated repair, the larger K14 positive area, as well as the increased Ki67 positive cells. Macrophage dysfunction is an important characteristic of delayed wound healing in diabetes mellitus (21, 22). In this study, functional experiments revealed that miR-145-5p overexpression inhibited M1 macrophage polarization while promoting M2 polarization in RAW 264.7 macrophage cells. Importantly, the overexpression of miR-145-5p increased the number of M2 macrophages ($CD206^{+}$) in diabetic wound. It has been demonstrated that macrophages of M2 facilitate the resolution of inflammation and promote tissue remodeling by releasing growth cytokines and anti-inflammatory cytokines (10, 11). Therefore, the polarization of macrophage toward the M2 phenotype mediated by miR-145a-5p might be an important mechanism responsible for its therapeutic effects on diabetic wound repair. Additionally, the bioinformatics analysis of the downstream miRNAs of miR-145a-5p suggested that miR-145a-5p was involved in the regulation of signal transduction, cell differentiation, apoptosis, cell cycle, lipid metabolic process, etc. Wang et al. found that miR-145a-5p regulated microglial polarization and the production of inflammatory cytokines, in which the NF-κB pathway might be involved (17). Chen et al. reported that miR-145a-5p modulated the polarization of M2 macrophage by targeting PAK7 and regulating β-catenin signaling in hyperlipidemia (53). Moreover, miR-145a-5p regulates the differentiation of myoblasts, adipocytes, and T cells (15, 16, 54). However, the underlying mechanisms by which miR-145a-5p regulates macrophage polarization have not been elucidated. More work is needed to verify the downstream genes and related signaling pathways of miR-145a-5p in regulating macrophage polarization.

The other differentially expressed miRNAs identified in our study, such as miR-183-5p, miR-143-3p, miR-let-7c, miR-26a-5p, miR-27a-3p, miR-30e-5p, and miR-let-7b, were mainly involved in the regulation of cellular proliferation and inflammation. The functions of these miRNAs were found to be chiefly as follows: First, miR-183-5p transferred to macrophages by exosomes promoted the secretion of proinflammatory cytokines (55); second, miR-143-3p participated in the regulation of LPS induced inflammation (56); third, miR-let-7c inhibited proinflammatory cytokine production in osteoarthritis and rheumatoid arthritis synovial fibroblasts (57); fourth, miR-26a-5p was capable of protecting against retinal neuronal impairment in diabetic mice by down-regulating phosphatase and tensin homolog (PTEN) (58); fifth, miR-27a-3p attenuated LPS-induced HK-2 cell apoptosis by downregulating the protein levels of TLR4 and NF-kappaB (59, 60); sixth, miR-30e-5p alleviated inflammation and cardiac dysfunction after myocardial infarction through targeting PTEN (61, 62); and seventh, miR-let-7b regulated the expression of inflammation-associated genes in monocytes, macrophages, and neutrophil (63, 64). The literature on the biological roles of miR-1a-3p and miR-1b-5p has been scanty. While these miRNAs might play important roles in the process of wound healing through the regulation of inflammation and cell proliferation, the effects of these identified miRNAs on wound healing as well as the underlying mechanisms remain to be further explored, where potential therapeutic targets, for instance, diabetic trauma, are expected to be pinpointed for chronic wound healing.

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 at: https://datadryad.org/stash/dataset/, doi: 10.5061/dryad.n5tb2rbwx.

ETHICS STATEMENT

The animal study was reviewed and approved by the Institutional Animal Care and Use Committee of Beijing Institute of Radiation Medicine.

AUTHOR CONTRIBUTIONS

YLi and LY: conceptualization. YH, HZ, YLiu, YY, CY, HY, YX, ZJ, and CH: methodology. YH and JW: formal analysis and writing—original draft preparation. YLi: writing—review and editing and supervision. LY: project administration and funding acquisition. All authors have read and agreed to the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2021.775523/full#supplementary-material

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