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Insights in inflammation 2022

Edited by
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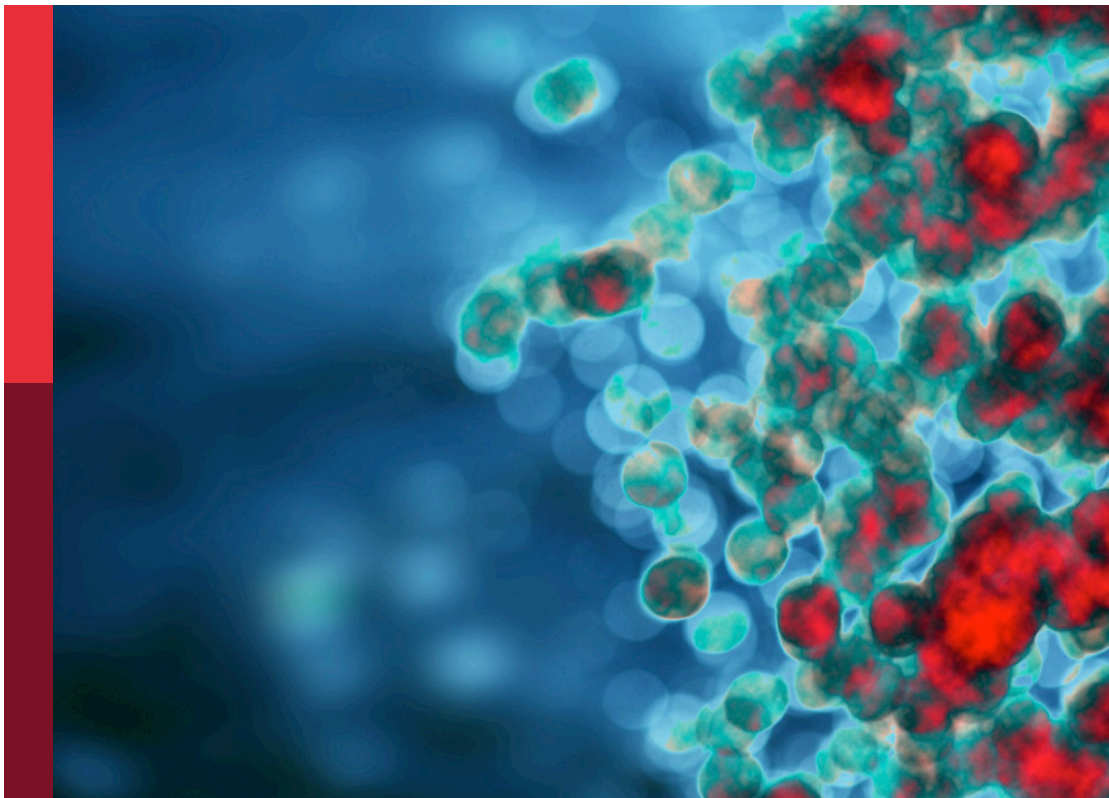

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Insights in inflammation: 2022

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Editorial: Insights in inflammation: 2022

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Editorial on the Research Topic
Insights in inflammation: 2022

This Research Topic is focused on new insights, novel developments, current challenges, latest discoveries, recent advances, and future perspectives in the field of Therapeutics. Most manuscripts in this section deal with the inflammatory component of disease which include specific musculoskeletal disease (osteoartritis, ankylosing spondylitis), other specific conditions (nasal polyps, asthma, atopic dermatitis, liver ischemia/reperfusion, burn and sepsis). The pathways and mechanisms investigated were related to signalling mechanisms (STAT3, FK506-binding proteins), inflammatory cytokines, receptors (prostanoid receptor CRTH2 and the scavenger receptor MSR1), transcription factors (Nrf2 and HIF-1a) and processes related to cell death (ferroptosis, autophagy, efferocytosis).

The cytokine signalling pathway involving JAK/STAT is a validated pharmacological target and JAK inhibitors are now approved for some chronic inflammatory disease. Yang et al. review the role of STAT3 in liver ischemia/reperfusion injury describing a not widely known effect on lipid metabolism. Tomiaki et al., using FK506 as a tool in a model of allergen-mediated airway inflammation, investigate the signalling mechanism of group 2 innate lymphoid cells (ILC2s) and type 2 helper T (TH2) cells in asthma.

A review by Gudgeon et al. highlights the importance of Macrophage scavenger receptor 1 (MSR1) as a potential biomarker and pharmacological target in inflammatory disease and cancer. This is one of the scavenger receptors that facilitate the uptake of lipoproteins by macrophages and hence the formation of foam cells. The review discusses MSR1 mutations, transcriptional regulation and signalling pathway and puts these in context with its possible role in innate immunity, atherosclerosis and diseases associated with inflammation.

A research paper by Chen et al. studied the expression of chemoattractant receptor-homologous molecule expressed on Th2 cells (CRTH2) in nasal polyps and found an association with eosinophil infiltration, which could be important as CRTH2 antagonists are being tested in clinical trials for other disease conditions.

Wang and He review the anti-inflammatory mechanisms mediated by Nrf2, and its interaction with pro-inflammatory transcription factors such as NFkB, in the context of osteoarthritis. This is a research field that may have clinical relevance given the poor response of osteoarthritis to TNF neutralizing agents and the fact that Nrf2 activators are already marketed for other pathologies (e.g. dimethylfumarate in multiple sclerosis). In a research paper, Sun et al. investigate the therapeutic effect of cold plasma in a mouse model of atopic dermatitis as well as in human keratinocytes *in vitro*. Their study identifies a mechanism by which cold plasma treatment induces the protein mesencephalic astrocyte-derived neurotrophic factor (MANF) that would then reduce inflammation and endoplasmic reticulum stress, possibly by increasing HIF-1 α at the transcriptional level.

Long et al. performed a pre-registered systematic review with meta-analysis of studies on the efficacy of iguratimod, an anti-inflammatory drug approved in China and Japan, on ankylosing spondylitis. The results indicate an efficacy of the drug in association with standard therapy. However, using a risk-of-bias tool they find that the evidence was judged to be moderate to very low, which should encourage clinicians to perform higher-quality trials.

Another approach represented in this Research Topic is that of genetic association. Liu et al. studies the association of variants in the IL-17 family genes with susceptibility to human diseases and finds an association with several diseases, including musculoskeletal disease, asthma and cancer. This study may be clinically relevant as anti-IL-17 antibodies are already approved for some pathologies.

Three reviews dealt with cell death. Zhang et al. reviews ferroptosis, a form of cell death regulated by iron, with a particular focus on osteoclasts and osteoblasts and its potential relevance in musculoskeletal disease. The review by Saas et al. deals with efferocytosis, a form of phagocytosis of apoptotic neutrophils by macrophages. They describe the various molecular mechanisms of efferocytosis and its role as an active mechanism involved in the resolution of inflammation. Autophagy is a process that is frequently, but not necessarily, associated with cell death and consists of the degradation of cellular components. Zhao et al. discuss the molecular mechanisms and pathways that regulate autophagy with a focus on sepsis-induced acute kidney injury.

Finally, research by Mulder et al. studies in depth the inflammatory response in burn wound tissue (eschar) from patients. By using immunohistochemistry, flow cytometry and immunoassays, the authors characterize the local infiltration of immune cells in the wound and correlate it with the expression of several cytokines.

Author contributions

PG wrote the original manuscript. All authors edited and approved the manuscript.

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.

Publisher's note

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ORIGINAL RESEARCH
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Cold Plasma Irradiation Attenuates Atopic Dermatitis *via* Enhancing HIF-1α-Induced MANF Transcription Expression

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Cold atmospheric plasma has been widely applied in medical treatment clinically, especially skin diseases. However, the mechanism of cold atmospheric plasma on the treatment of skin diseases is still undefined. In this study, dinitrofluorobenzene-induced atopic dermatitis mice model was constructed. Cold atmospheric plasma was able to decrease skin cells apoptosis, relieve skin inflammation, ER stress and oxidative stress caused by dinitrofluorobenzene stimulation, which was mediated by cold atmospheric plasma-induced MANF expression. In terms of mechanism, hypoxia-inducible factor-1α expression was increased intracellularly after cold atmospheric plasma treatment, which further bound to the promoter region of *manf* gene and enhanced MANF transcriptional expression. This study reveals that cold atmospheric plasma has a positive effect on atopic dermatitis treatment, also demonstrates the regulatory mechanism of cold atmospheric plasma on MANF expression *via* HIF-1α, which indicates the potential medical application of cold atmospheric plasma for atopic dermatitis treatment.

Keywords: cold atmospheric plasma, mesencephalic astrocyte-derived neurotrophic factor, hypoxia-inducible factor-1α, nuclear factor kappa-B, atopic dermatitis

INTRODUCTION

Atopic dermatitis (AD) is a chronic disease of skin with characteristics of relapse and skin inflammation, which has a rising incidence worldwide (1). AD's pathogenesis is very complex to be barely defined currently. Multiple factors have been reported to be closely associated with the occurrence and development of AD, including immune dysfunction, skin function failure and environmental changes (2). It has been proven that Endoplasmic Reticulum Stress (ER stress) and oxidative stress responses play a pivotal pathogenic role for AD (3, 4). At present, AD's therapy is mainly based on corticosteroid hormone for skin coating treatment, but the long-term use of hormone therapy is greatly possible to trigger dyslipidemia, dysarteriotony and glucose

Abbreviations: AD, Atopic dermatitis; CAP, Cold atmospheric plasma; MANF, Mesencephalic astrocyte-derived neurotrophic factor; HIF-1, Hypoxia-inducible factor-1; DNFB, dinitrofluorobenzene; TM, Tunicamycin; ROS, Reactive oxygen species; NF-κB, Nuclear factor kappa-B.

abnormality, also lead to the excessive loss of calcium (5, 6). It is urgent to find a new and effective therapy for AD treatment with less even no side effects.

Cold atmospheric plasma (CAP) is a sort of ionized gas at the room temperature level that is composed of massive active particles like ions, electrons, free radicals, reactive oxygen species (ROS) and nitrogen species (7). CAP has been widely involved in medical applications. CAP is able to promote acute and chronic wound healing, improve oral cleaning and disinfection, facilitate cancer treatment (8–10). It has been demonstrated that CAP up-regulates the expression of hypoxia-inducible factor-1 (HIF-1) in human dermal fibroblasts (11, 12). HIF-1 is a heterodimer consisting of HIF-1α and HIF-1β monomers, which has been found to be highly expressed in skin injury, hypoxia and radiotherapy response (13, 14).

Mesencephalic astrocyte-derived neurotrophic factor (MANF) is a member of neurotrophic factor family to exert the protective effect on neurons and some non-neuronal cells (15–18). Also, in response to ER stress, MANF expression is up-regulated as one of ER stress-related proteins (19). Recently, more research evidences have demonstrated that MANF plays an anti-inflammatory role in some acute and chronic inflammatory diseases (17, 18, 20), which is mediated by binding to NF-κB p65 for impeding p65 nuclear translocation, further negatively affecting NF-κB signal activation (21). Although MANF's inflammation inhibitory effect has been clearly verified, there is still no experimental finding to define the relationship between MANF and skin inflammation.

In this study, dinitrofluorobenzene (DNFB)-induced AD mice model was constructed to explore the effects of CAP and MANF on AD progress. Moreover, using human immortal keratinocyte line (HaCaT) *in vitro*, the transcriptional regulatory mechanism of HIF-1α induced by CAP on MANF expression was studied. These research results suggest the clinical application potential of CAP on AD treatment and prevention, also preliminarily reveal HIF-1α-mediated MANF transcriptional regulation.

METHOD DETAILS

DNFB-Induced AD Mice Model

6-8 weeks C57BL/6J mice were depilated on the skin of back (Area: 3 cm^2). 0.5% DNFB in the mixed solution of acetone and olive oil (3:1) was used for coating mice's depilated area every three days for four times. For CAP treatment, DNFB-induced AD mice were treated by CAP for 3 minutes. For hrMANF or MANF antibody treatment, DNFB-induced AD mice were injected subcutaneously by hrMANF protein (0.5 mg/kg) or MANF antibody (600μg/kg). Mice breeding was operated in SPF-class animal laboratory. All animal experiments were performed according to protocols approved by the Animal Ethics Committee of Anhui Medical University (Approval number: LLSC20210791).

HaCaT Cell Culture and CAP Treatment

The human immortal keratinocyte HaCaT cell line was cultured in Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal bovine serum (FBS). The culture condition was 37°C and 5% CO_2. CAP was produced by an atmospheric pressure dielectric barrier discharge jet plasma source mainly consisting of a quartz tube (inner diameter 4 mm) and a cooper ring. The cooper ring was powered by a high-voltage power supply generating a sinusoidal voltage waveform with 8.25 kV peak value at a frequency of 10 kHz. Helium (flow rate: 400 sccm) and oxygen (flow rate: 4 sccm) were mixed to introduce into the quartz tube. For mice skin treatment, the skin inflammation area was involved in CAP treatment for 3 minutes. For HaCaT cell treatment *in vitro*, HaCaT cell nutrient solution was firstly treated by CAP for 30 seconds, which was next used to cultivate HaCaT cells for overnight.

Antibodies and Reagents

Antibodies involved in this study contain: anti-Cleaved caspase3 (Abcam, ab32042); anti-HMGB1 (Abcam, ab79823); anti-TNF-α (Abcam, ab183218); anti-IL-1β (Abcam, ab9722); anti-CCL2 (Abcam, ab25124); anti-Bip (Abcam, ab21685); anti-CHOP (Abcam, ab11419); anti-HO-1 (Abcam, ab52947); anti-MANF (Abcam, ab67271); anti-GAPDH (Abcam, ab3285); anti-HIF-1α (Abcam, ab243860); Anti-CD163 (Abcam, ab182422); Goat Anti-Rabbit IgG H&L (HRP) (Abcam, ab6721); PE anti-CD11b (Abcam, ab25533); APC anti-Ly6C (Abcam, ab93550); Alexa Flour 488 anti-Ly6G (Abcam, ab283276). The involved reagents contain: DNFB (Sigma, St Louis, MO, USA, 42085); hrMANF protein (Abcam, ab123227); Goat Anti-Mouse/Rabbit Polymer Immunohistochemistry Detection Kit (ZSGB-BIO, PV-6000); Lipofectamine™ 3000 (Thermo Fisher, L3000150).

Immunohistochemistry

Mice's skin tissues (n=5) were used for Immunohistochemistry according to the previous research (20). Skin tissues were fixed in 10% formaldehyde. Paraffin sections were produced after paraffin embedding, then deparaffinization in dimethylbenzene. Rehydration was performed in 100%, 90%, 80% and 70% ethanol for 5 minutes respectively. Hematoxylin and eosin were used for hematoxylin-eosin (HE) staining. After rinse, paraffin sections were performed by tissue antigen recovery, followed by heating and 1×PBS rinse. Peroxidase blocking agent was used for incubation at 37°C for 30 minutes. After 1×PBS rinse, paraffin sections were incubated with the goat serum at 37°C for 30 minutes. Then, the corresponding antibodies were used for incubation at 4°C overnight. After secondary antibody incubation at 37°C for 30 minutes and 1×PBS rinse, 3, 3′-diaminobenzidinetetrahydrochloride (DAB) and hematoxylin staining were performed. For immumohistochemical staining, paraffin sections were stained by antibodies of Cleaved caspase3 (1/300), HMGB1 (1/300), TNF-α (1/400), IL-1β (1/400), CCL2 (1/400), Bip (1/500), CHOP (1/300), HO-1 (1/1000), MANF (1/200), CD163 (1/400) and HIF-1α (1/400). Images were obtained by Olympus Microscope BX53.

Western Blot

The reduced sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE, 12%) was performed to separate protein samples extracted from mice's skin tissues (n=5) and

cells. Each protein sample was 10 μg. After SDS-PAGE, PVDF membrane (0.45μm, 26.5 cm x 3.75 m) was used for protein transfer, followed by 5% BSA, primary antibodies (anti-Cleaved caspase3, 1/500; anti-HMGB1, 1/20000; anti-TNF-α, 1/1000; anti-IL-1β, 1/1000; anti-CCL2, 1/1000; anti-Bip, 1/2000; anti-CHOP, 1/800; anti-HO-1, 1/2000; anti-MANF, 1/1000; anti-GAPDH, 1/5000; anti-HIF-1α, 1/1000; anti-CD163, 1/1000) and second antibodies (1/4000) incubation. Images were obtained by Chemiscope 6000 pro touch imaging system.

Real Time-Quantitative Polymerase Chain Reaction

Total RNA extraction from mice's skin tissues (n=5) was obtained by Trizol reagent, and reverse transcription was performed by PrimeScript RT reagent Kit (TaKaRa Bio, Dalian, China) according to manufacturer's instruction. The involved primers contain: TNF-α, forward 5′-CAGGAGGGAGAACAGAA ACTCCA-3′ and reverse 5′-CCTGGTTGGCTGCTTGCTT-3′; HMGB1, forward 5′-GCTGACAAGGCTCGTTATGAA-3′ and reverse 5′-CCTTTGATTTTGGGGCGG

TA-3′; IL-1β, forward 5′-GAAATGCCACCTTTTGACA GTG-3′ and reverse 5′-TGGATGCTCTCATCAGGACAG-3′; CCL2, forward 5′-TAAAAACCTGGATCG

GAACCAAA-3′ and reverse 5′- GCATTAGCTTCAGATTT ACGGGT-3′; Bip, forward 5′-ACTTGGGGACCACCT ATTCCT-3′ and reverse 5′-GTTGCCCTGATCG

TTGGCTA-3′; CHOP, forward 5′- AAGCCTGGTATGAG GATCTGC-3′ and reverse 5′-TTCCTGGGGATGAGATAT AGGTG-3′; HO-1, forward 5′-AGGTACACATCCAA

GCCGAGA-3′ and reverse 5′-CATCACCAGCTTAAAGC CTTCT-3′; MANF, forward 5′-TCTGGGACGATTTTAC CAGGA-3′ and reverse 5′-CTTGCTTCACGGC

AAAACTTT-3′; CD163, forward 5′-GGTGGACACA GAATGGTTCTTC-3′ and reverse 5′-CCAGGAGCGTTA GTGACAGC-3′; GAPDH, forward 5′-AGGTCGGTG

TGAACGGATTTG-3′ and reverse 5′-GGGGTCGTTGATG GCAACA-3′. The 2^-ddCT calculation was used.

Enzyme-Linked Immunosorbent Assay

The serum from mice (n=8) was collected to examine the serum levels of TNF-α, IL-1β and IL-10. The involved ELISA kits contain: Mouse TNF-α *in vitro* SimpleStep ELISA Kit (Abcam, ab208348); Mouse IL-1 beta *in vitro* SimpleStep ELISA Kit (Abcam, ab100704); Mouse IL-10 *in vitro* SimpleStep ELISA Kit (Abcam, ab255729). ELISA was performed according to manufacturer's instructions.

Terminal Deoxynucleotidyl Transferase dUTP Nick end Labeling Assay

Paraffin-embedded mice skin tissues (n=5) were prepared for TUNEL assay. *In Situ* Cell Death Detection Kit, Fluorescein (Roche, Basel, Switzerland, 11684795910) was used according to manufacturer's instruction. The final results were acquired by Olympus Microscope BX53/IX71.

ROS and NO Examination

The serum from mice (n=8) was collected to examine the serum ROS and NO levels. Total Reactive Oxygen Species and Nitric Oxide Assay Kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, Jiangsu, China) was used for ROS and NO examination according to manufacturer's instruction.

Flow Cytometry

Peripheral blood samples from mice were used for flow cytometry assay. Cells in peripheral blood were blocked by 1% mouse serum, then antibody incubation was performed for 30 minutes. After PBS washing, $CD11b^{+}Ly6C^{hi}$ and $CD11b^{+}Ly6G^{+}$ immune cells in peripheral blood were analyzed by BD FACS Verse.

Chromatin Immunoprecipitation

HaCaT cells were processed according to previous protocol (22). Anti-HIF-1α and normal IgG antibodies were separately added into lysates. After CHIP assay, PCR was performed for HIF-1α-MANF promoter binding analysis. Human MANF-pro-HIF-1α primers: forward 5'-CAACGGTTCCCGCATCCTG-3' and reverse 5'-CTGAATCGTGGCTTGGTGG-3'.

Dual-Luciferase Reporter Assey

HaCaT cells were co-transfected with luciferase reporters of pGL3-MANF promoter control or pGL3-MANF promoter HIF-1α binding site mutation plasmid together with pcDNA-control or pcDNA-HIF-1α mutation plasmid, followed by cell culture for 24 hours. Cell lysate was extracted to examine the luciferase activity by Dual-Luciferase Reporter Assay System (Promega, USA). Renilla luciferase activity was used for normalization.

Statistical Analysis

Data are presented as means ± SD. Two-way ANOVA was used for statistical comparison. p *value*<0.05 indicates significant difference. An asterisk (*), two asterisks (**) and three asterisks (***) stand for $p<0.05$, $p<0.01$ and $p<0.001$ respectively. For mice experiments, 8 mice per group (n=8) were used. All experiments were performed independently at least three times.

RESULTS

CAP Treatment Weakened DNFB-Induced Apoptosis to Relieve Skin Injury in Mice

To clarify the effect of CAP on AD, we constructed DNFB-induced AD mice model according to the previous reports (23, 24). **Figure 1A** showed that DNFB was able to promote skin thickening, induce hyperkeratosis and parakeratosis, increase the skin tissue infiltration of inflammatory cells. Comparatively, although CAP treatment alone could not induce skin injury and cell apoptosis in mice (**Figures S1A, B**), CAP treatment greatly weakened DNFB-induced skin injury in mice (**Figure 1A**). Caspase 3 is one of the classic apoptosis-associated proteins (25). IHC and WB results showed that cleaved caspase 3 was remarkably up-regulated in DNFB-induced mice skin tissues. After CAP treatment, the increase of cleaved caspase 3 induced by DNFB was restrained (**Figures 1A, B** and **S1C, D**). Moreover, TUNEL assay results in **Figures 1A** and **S1C** showed that CAP treatment was able to decrease DNFB-induced skin cell apoptosis.

These evidences suggest CAP treatment relieves DNFB-induced apoptosis in skin tissues of mice, finally decreasing DNFB-induced skin injury.

CAP Treatment Attenuated DNFB-Induced Skin Inflammation, ER Stress and Oxidative Stress in Mice

The inflammatory response is often accompanied by ER stress and oxidative stress responses (26). Accordingly, we examined the commonly-used indicators of inflammation, ER stress and oxidative stress, including Tumor Necrosis Factor-α (TNF-α), Interleukin-1β (IL-1β) (27), High Mobility Group Box 1 (HMGB1) (28), Chemokine CCL2 (29), Glucose Regulated Protein 78 (GRP78, also known as Bip), CCAAT/enhancer binding protein homologous protein (CHOP) (30), Heme Oxygenase-1 (HO-1) (31) and MANF. IHC, WB and RT-qPCR results showed that DNFB stimulation could promote expressions of pro-inflammatory cytokines TNF-α and IL-1β, Chemokine CCL2, pro-inflammatory HMGB1, ER stress-related proteins Bip and CHOP, oxidative stress-related protein HO-1 in skin tissues of mice, indicating DNFB-induced AD mice had the greater inflammation, ER stress and oxidative stress responses compared with untreated mice; also, CAP treatment partly alleviated DNFB-induced skin inflammation, ER stress and oxidative stress (**Figures 2A–C** and **S2A, B**). Consistently, DNFB-induced AD mice had the higher serum levels of TNF-α, IL-1β, ROS and NO, but the lower serum anti-inflammatory IL-10. CAP treatment significantly inhibited the serum TNF-α, IL-1β, ROS and NO, also promoted the serum IL-10 (**Figure 2D**). In the AD process, it has been found that pro-inflammatory immune cells were increased in peripheral blood (32, 33). As shown in **Figure 2E**, after DNFB stimulation, the proportions of $CD11b^{+}Ly6C^{hi}$ monocytes and $CD11b^{+}Ly6G^{+}$ neutrophils in peripheral blood of mice were greatly increased, which were inversely lowered by CAP treatment. By M2-type macrophage marker CD163 detection in **Figure S2C and D**, the skin M2 macrophage differentiation was greatly promoted by CAP treatment in DNFB-induced AD mice. Moreover, we found that DNFB slightly stimulated MANF transcription and expression in skin tissues of mice, which was further greatly promoted by CAP treatment (**Figures 2A–C** and **S2A, B**). The above data suggest that DNFB-induced skin inflammation, ER stress and oxidative stress are weakened by CAP treatment that largely up-regulates MANF expression.

CAP Treatment Enhanced MANF Expression to Reduce DNFB-Induced Skin Inflammatory Injury, ER Stress and Oxidative Stress in Mice

Next, we studied whether CAP treatment suppressed AD occurrence and development *via* MANF up-regulation. The exogenous human recombinant MANF (hrMANF) protein and MANF antibody were used to treat DNFB-induced AD mice. The treatment of CAP, hrMANF and MANF antibody alone did not significantly affect skin integrity, inflammation, ER stress and oxidative stress of mice (**Figures S3A–C**). As shown in **Figures 3A–D** and **S4A, B**, hrMANF treatment significantly relieved DNFB-induced skin inflammation, ER stress and oxidative stress, which was consistent with the effect of CAP treatment. However, inhibiting MANF protein *via* MANF

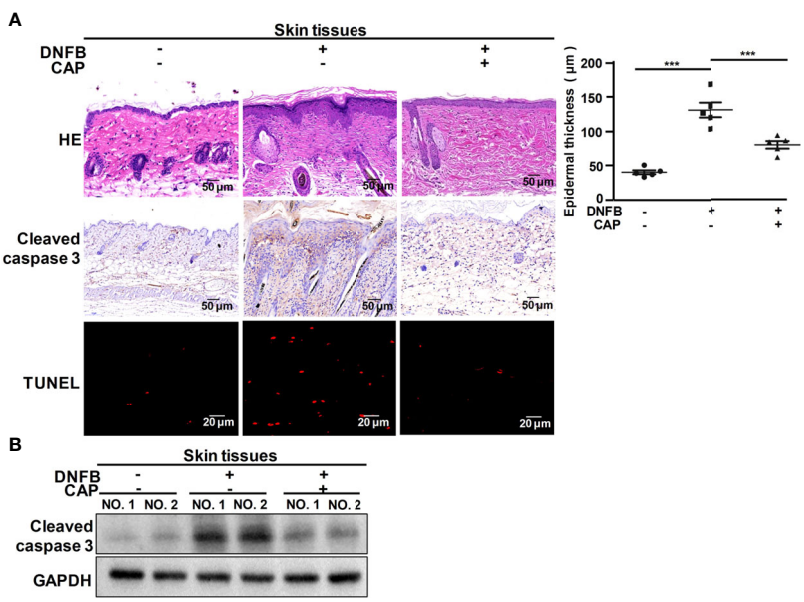

FIGURE 1 | CAP alleviated DNFB-induced skin injury *via* attenuating apoptosis. DNFB-induced AD mice model was constructed, followed by CAP treatment, n=8. Skin tissues (n=5) were used for HE, immunohistochemical staining of cleaved caspase 3 and TUNEL assay **(A)**, as well as western blot of cleaved caspase 3 **(B)**. Epidermal thickness was evaluated. GAPDH serves as control for normalization. Data are expressed as mean ± SEM. ***$p < 0.001$.

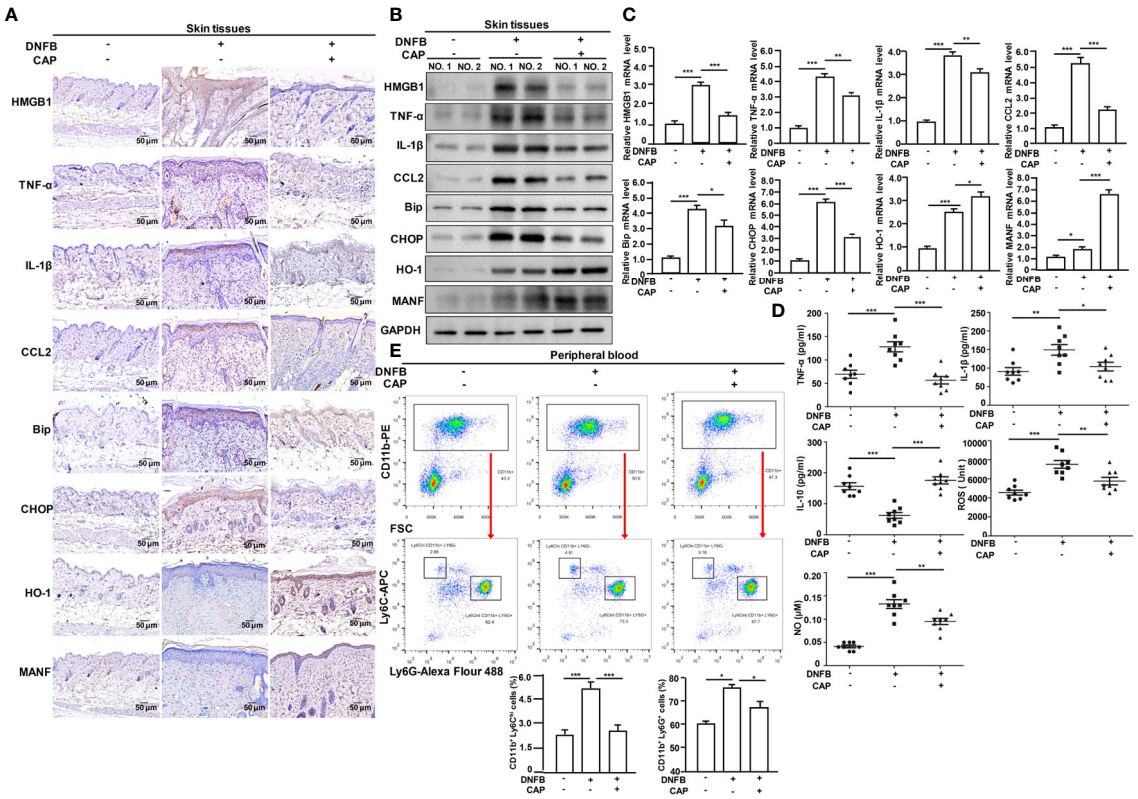

FIGURE 2 | CAP reduced DNFB-induced skin inflammation, ER stress and oxidative stress in mice. DNFB-induced AD mice model was constructed, followed by CAP treatment, n=8. Skin tissues (n=5) were used for immunohistochemical staining of HMGB1, TNF-α, IL-1β, CCL2, Bip, CHOP, HO-1 and MANF **(A)**, as well as western blot **(B)** and RT-qPCR **(C)** of the indicated proteins. GAPDH serves as control for normalization. **(D)** Serum samples (n=8) were used for ELISA of TNF-α, IL-1β, IL-10, NO and ROS. **(E)** $CD11b^{+}Ly6C^{hi}$ and $CD11b^{+}Ly6G^{+}$ cells were examined by flow cytometry. Data are expressed as mean ± SEM. *p < 0.05, **p < 0.01, *** p < 0.001.

antibody could partly resist the protective effect of MANF against DNFB-induced skin inflammation injury, ER stress and oxidative stress in mice, indicating the protective effect of CAP against DNFB-induced skin inflammation injury was mediated by CAP-induced MANF up-regulation. Therefore, MANF expression induced by CAP treatment plays an important role in CAP-mediated inhibitory effect on DNFB-induced skin inflammation, ER stress and oxidative stress responses.

CAP treatment Induced MANF Transcriptional Expression *via* Increasing HIF-1α Level

Furthermore, we explored the specific mechanism on CAP-mediated MANF transcriptional regulation. It has been reported that CAP is able to increase HIF-1α expression (11). We also found that CAP treatment alone could slightly increase HIF-1α and MANF levels in skin tissues of mice (**Figures S5A, B**). We have previously found that there is a potential HIF-1α binding site in the promoter region of human *manf* gene (From +357 to +365), suggesting the possibility of HIF-1α-mediated direct transcriptional regulation for MANF expression. IHC results in **Figure 4A** showed that DNFB stimulated HIF-1α expression, and CAP treatment further increased HIF-1α level in skin tissues of mice. In **Figure 4B** and **Figure S5C**, CAP treatment could significantly promote HIF-1α and MANF expressions in skin tissues of mice in a time-dependent way. To clarify the interplay among CAP, HIF-1α and MANF expression, we performed a series of experiments *in vitro* by using HaCaT cells. We conducted HIF-1α gene silencing by two different HIF-1α siRNA sequences in HaCaT cells. After HIF-1α expression was down-regulated, the intracellular MANF level was decreased consequently (**Figure 5A** and **Figure S5D**). Also, HIF-1α mutant protein expression plasmid (pcDNA-HIF-1α Mut) was constructed to restrain HIF-1α degradation. As shown in **Figure 5B** and **Figure S5E**, after HIF-1α mutant protein was over-expressed in HaCaT cells, MANF expression was greatly promoted. These data indicate there is a positive correlation between HIF-1α and MANF expression in both mice skin tissues and HaCaT cells. CHIP result in **Figure 5C** showed that HIF-1α could bind to *manf* gene promoter region after CAP treatment. Over-expression of HIF-1α mutant protein was able to markedly enhance *manf* promoter's activity, but HIF-1α binding site mutation in *manf* promoter eliminated HIF-1α-mediated MANF transcriptional activation (**Figure 5D**). Altogether, CAP treatment induces the expression

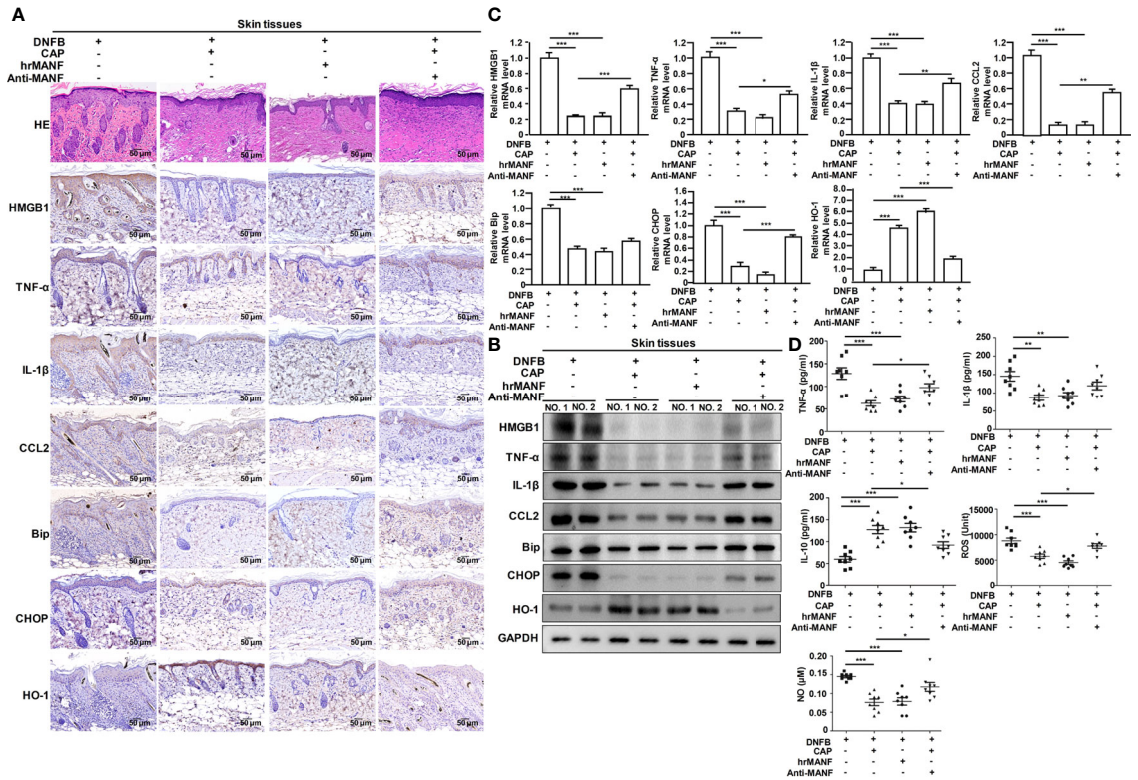

FIGURE 3 | CAP relieved DNFB-induced skin inflammatory injury, ER stress and oxidative stress *via* promoting MANF expression. DNFB-induced AD mice model was constructed, followed by CAP treatment, hrMANF treatment and MANF antibody treatment, n=8. Skin tissues (n=5) were used for HE and immunohistochemical staining of HMGB1, TNF-α, IL-1β, CCL2, Bip, CHOP and HO-1 **(A)**, as well as western blot **(B)** and RT-qPCR **(C)** of the indicated proteins. GAPDH serves as control for normalization. **(D)** Serum samples (n=8) were used for ELISA of TNF-α, IL-1β, IL-10, NO and ROS. Data are expressed as mean ± SEM. *p < 0.05, **p < 0.01, *** p < 0.001.

of HIF-1α that directly binds to *manf* promoter region for MANF transcriptional activation.

DISCUSSION

Atopic dermatitis is a sort of common skin disease with the characteristic of chronic inflammation to show the systemic disorder, further progressing to asthma, allergic rhinitis and other diseases (34). DNFB is a commonly-used chemical reagent to induce AD-like skin inflammation and injury (35). There are some previous reports and researches that use DNFB-induced mouse skin inflammation as allergic contact dermatitis and atopic dermatitis models (36–39). In this study, we used DNFB-induced AD mice model to reveal the effect of CAP on the pathological process of AD. Our findings indicate that CAP exerts an inhibitory effect on DNFB-induced AD-like skin inflammatory injury, ER stress and oxidative stress responses in mice. Currently, the clinical therapy for AD is mainly based on corticosteroid hormone, which gives rise to some side effects (6). In light of CAP's negative effect on AD, it is potential to involve CAP in the clinical treatment of AD without significant side effects. In our previous research (40), we have found that CAP is able to decrease the human non-small cell lung carcinoma A549 cell inflammation and oxidant stress induced by Tunicamycin. The transitory and low-intensity CAP treatment only induces a degree of ROS increase, not the overwhelming ROS production. Consistently, we speculate that the slight ROS production induced by CAP in our study may exert the anti-inflammatory and antioxidative effect *via* amplifying the correlated signaling pathways.

For the mechanism on how CAP restrains DNFB-induced skin inflammation and injury, our study suggests CAP-induced MANF expression in skin tissues plays a key role to mediate CAP's protective effect against DNFB-induced AD in mice. Without CAP treatment, DNFB stimulation purely is able to slightly increase MANF level in skin tissues. Interestingly, MANF transcriptional expression is significantly promoted by CAP treatment. MANF has been proven to exert the anti-inflammatory effect in multiple inflammation-linked diseases, like acute kidney injury (17), bacterial myocarditis (18) and antigen-induced arthritis (21). This study further expands MANF's anti-inflammatory role in atopic dermatitis, possibly other skin inflammatory diseases. In addition, there are some researches demonstrating MANF's moderating effects on

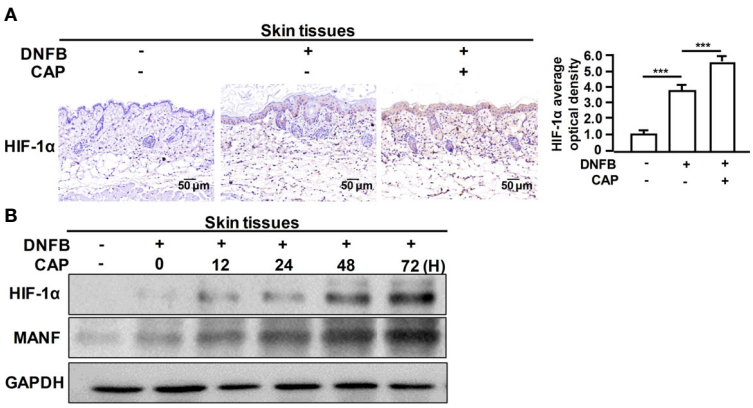

FIGURE 4 | CAP treatment significantly enhanced DNFB-induced HIF-1α expression in skin tissues. DNFB-induced AD mice model was constructed, followed by CAP treatment, n=8. **(A)** Skin tissues (n=5) were used for immunohistochemical staining of HIF-1α. The average optical density was analyzed. **(B)** At 0, 12, 24, 48 and 72 hours after CAP treatment, skin tissues (n=5) were used for western blot of HIF-1α and MANF. GAPDH serves as control for normalization. Data are expressed as mean ± SEM. ***$p < 0.001$.

functional differentiation of macrophages (41). Overall, macrophages are mainly divided into two different functional subtypes: pro-inflammatory M1 macrophages and anti-inflammatory M2 macrophages (42). Joana Neves et al. have found that MANF is able to induce $YM^{+}Arg^{+}$ M2 anti-inflammatory macrophage polarization in an autocrine way for retinal damage repair (41). Also, mono-macrophage-specific MANF deficiency significantly affects M1/M2 differentiation of splenic macrophages in the hepatic fibrosis process (20). We examined pro-inflammatory immune cells in peripheral blood of mice, then found that CAP treatment could partly suppress the increased proportion of pro-inflammatory $CD11b^{+}Ly6C^{hi}$

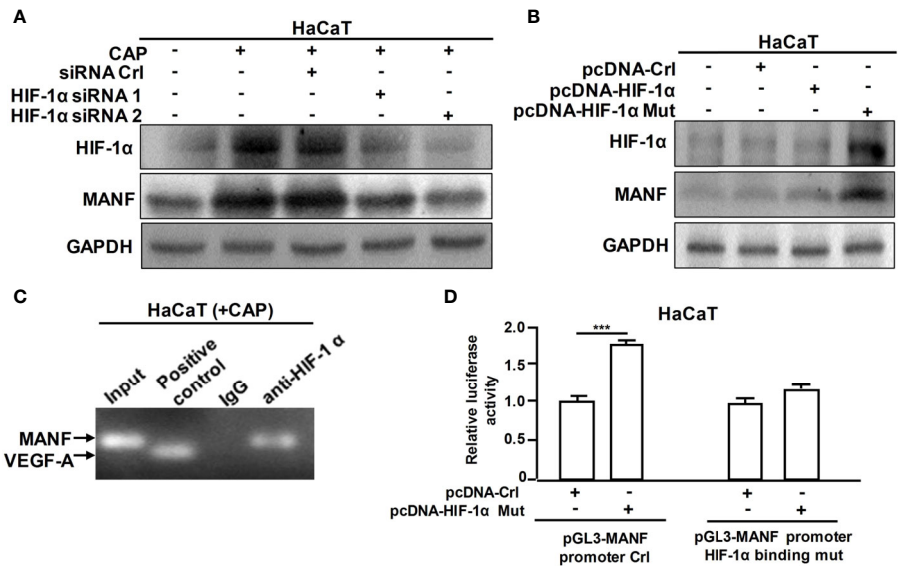

FIGURE 5 | CAP induced MANF transcription and expression by HIF-1α-mediated transcriptional regulation. **(A)** HaCaT cells transfected by siRNA control, HIF-1α siRNA 1 and HIF-1α siRNA 2 respectively were treated by CAP, followed by western blot of HIF-1α and MANF. **(B)** HaCaT cells transfected by pcDNA-control, pcDNA-HIF-1α and pcDNA-HIF-1α mutation plasmid respectively were treated by CAP, followed by western blot of HIF-1α and MANF. GAPDH serves as control for normalization. **(C)** HaCaT cells treated by CAP were used for Chromatin Immunoprecipitation assay. HIF-1α antibody was used for HIF-1α protein immunoprecipitation. IgG antibody serves as negative control. VEGF-A was involved as a positive control for HIF-1α binding. **(D)** HaCaT cells transfected by pcDNA-control and pcDNA-HIF-1α mutation plasmid respectively were used for dual-luciferase reporter assay of pGL3-MANF promoter control or pGL3-MANF promoter HIF-1α binding site mutation plasmid. The relative luciferase activity was analyzed. All experiments were performed independently at least three times. Data are expressed as mean ± SEM. ***$p < 0.001$.

monocytes and $CD11b^{+}Ly6G^{+}$ neutrophils in peripheral blood induced by DNFB stimulation, as well as promote M2 anti-inflammatory macrophage differentiation *via* CD163 detection. In the future, we plan to analyze the change of macrophages' M1/M2 differentiation in skin tissues and peripheral blood of mice after CAP treatment. Besides MANF, there are many other target genes transcriptionally regulated by HIF-1α, including some anti-apoptosis genes (43), which may mediate CAP's protective effect on DNFB-induced AD.

As an ER stress-related protein, MANF has been demonstrated to be up-regulated *via* the direct binding of XBP1s to ER stress response elements in MANF promoter region (44). In this study, we found that HIF-1α had a direct transcriptional regulation on MANF expression, and there was a verified HIF-1α binding site in MANF promoter. The previous researches have reported that MANF expression in glial cells is enhanced under the condition of focal cerebral ischemia (45); also, ischemia in heart is able to induce MANF expression as well (46). These evidences indicate that the ischemic and hypoxic environment contributes to MANF up-regulation, which may be attributed to ER stress response induced by ischemia-hypoxia (45–47). Therefore, two different pathways are involved in hypoxia-caused MANF up-regulation. Unfolded protein response (UPR) is often intensified by hypoxia to indirectly enhance MANF expression. Moreover, hypoxia improves HIF-1α protein stability *via* inhibition of HIF-1α degradation to increase the intracellular HIF-1α level (48), further promote HIF-1α-mediated MANF transcriptional expression. Besides hypoxia, our data showed that CAP treatment could raise HIF-1α level in the non-hypoxic condition. It has been found that mitochondrial-derived ROS is a non-hypoxic factor for HIF-1α stabilization and HIF-1 activation (49, 50). It is possible that ROS generated by CAP irradiation leads to the increase of HIF-1α in the non-hypoxic environment.

CONCLUSION

In this study, CAP treatment is able to protect against DNFB-induced skin inflammation, ER stress and oxidative stress of mice, further alleviate DNFB-induced mice skin injury. The protective effect of CAP on DNFB-induced AD mice model is mediated by CAP-induced MANF up-regulation. CAP promotes the increase of HIF-1α that binds to MANF promoter region for MANF transcriptional activation and expression.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The animal study was reviewed and approved by School of Basic Medical Sciences, Anhui Medical University, Hefei, China. Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

GN, DW and LF designed the research and wrote the manuscript. TS, XZ, CH, SY, LK, CL, YZ, and ZY performed the experiments, collected and analyzed the data. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.941219/full#supplementary-material

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Nrf2-mediated anti-inflammatory polarization of macrophages as therapeutic targets for osteoarthritis

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Macrophages are the most abundant immune cells within the synovial joints, and also the main innate immune effector cells triggering the initial inflammatory responses in the pathological process of osteoarthritis (OA). The transition of synovial macrophages between pro-inflammatory and anti-inflammatory phenotypes can play a key role in building the intra-articular microenvironment. The pro-inflammatory cascade induced by TNF-α, IL-1β, and IL-6 is closely related to M1 macrophages, resulting in the production of pro-chondrolytic mediators. However, IL-10, IL1RA, CCL-18, IGF, and TGF are closely related to M2 macrophages, leading to the protection of cartilage and the promoted regeneration. The inhibition of NF-κB signaling pathway is central in OA treatment *via* controlling inflammatory responses in macrophages, while the nuclear factor erythroid 2-related factor 2 (Nrf2) signaling pathway appears not to attract widespread attention in the field. Nrf2 is a transcription factor encoding a large number of antioxidant enzymes. The activation of Nrf2 can have antioxidant and anti-inflammatory effects, which can also have complex crosstalk with NF-κB signaling pathway. The activation of Nrf2 can inhibit the M1 polarization and promote the M2 polarization through potential signaling transductions including TGF-β/SMAD, TLR/NF-κB, and JAK/STAT signaling pathways, with the regulation or cooperation of Notch, NLRP3, PI3K/Akt, and MAPK signaling. And the expression of heme oxygenase-1 (HO-1) and the negative regulation of Nrf2 for NF-κB can be the main mechanisms for promotion. Furthermore, the candidates of OA treatment by activating Nrf2 to promote M2 phenotype macrophages in OA are also reviewed in this work, such as itaconate and fumarate derivatives, curcumin, quercetin, melatonin, mesenchymal stem cells, and low-intensity pulsed ultrasound.

KEYWORDS

Nrf2, macrophage, osteoarthritis, intra-articular, anti-inflammation

1 Introduction

Osteoarthritis (OA) is a highly prevalent musculoskeletal disorder characterized by pain, deformity, and functional deficits. Globally, it is a major medical and socioeconomic burden (1). Histologically, OA is characterized by cartilage degeneration, synovial lining thickening, and subchondral sclerosis (2). In pathophysiology, a low-grade, chronic inflammation predominantly leads to synovial joint deterioration as a result of an innate immune response (3). *In vivo* imaging evidence in patients with OA indicates a crucial role for activated macrophages, which is linked to its severity. Moreover, the disruption of macrophage transition may contribute to chronic and irreversible inflammatory changes in OA-affected joints (4). The activation of macrophages with heterogeneous phenotypes, which can exert pro- and anti-inflammatory effects on articular tissues during OA, has been suggested as a potential therapeutic target (5).

The macrophages within synovial joints include resident and interstitial subsets. The interstitial population of recruited monocyte-derived macrophages could exert functions of joint inflammation. While, a tight-junction-mediated shield composing of the subset of epithelial-like $CX3CR1^{+}$ tissue-resident macrophages could restrict the inflammatory response (6, 7). OA mainly results from innate immune response induced by macrophages that are marked by CD14 and F4/80 as general surface antigens (8, 9). Furthermore, the plastic heterogeneous phenotypes of macrophages include classically activated pro-inflammatory phenotypes (CD80, CD86, and CD11b as M1 surface markers) and alternatively activated anti-inflammatory phenotypes (CD163, and CD206 as M2 surface markers) (10–12). There are several subtypes of M2 macrophages, including M2a marked by CD206, M2b marked by CD86, M2c marked by CD163 (13, 14), and M2d marked by CD68 (15–17). Induced by IL-4 and IL-13, M2a plays a vital role in anti-inflammation response and wound-healing *via* up-regulating the expression of IL-1RA, IL-10, CCL-18, and TGF-β. M2b is the immune regulator between M1 and M2a, which serves in both pro-and anti-inflammation responses. Besides, IL-10, TGF-β, or glucocorticoids can induce M2c activation, which causes the secretion of IL-10, CCL-18, and TGF-β, and M2c also has a powerful phagocytosis function (18, 19). While, M2d, also called tumor-associated macrophages (TAMs), plays a vital role in potent immunosuppression, angiogenesis, wound healing, and cancer metastasis (15, 16). However, most experimental therapies or mechanisms for OA use the balance between M1 and M2 as a key point (5, 20–22). In this review, the polarization state of macrophages will be dichotomized without discussing specific subtypes of M2. In addition, synovia in OA patients has a substantial proportion of M1 macrophages, which is consistent with CD14 and CD163 expression levels (23). Macrophage phenotypes are influenced by intracellular redox metabolism, as evidenced by increasing studies, leading to metabolic reprogramming from glycolysis in M1 to oxidative phosphorylation (OxPhos) in M2 (5, 24–26).

Nuclear factor erythroid 2-related factor 2 (Nrf2) is a transcription factor expressed in most tissues and cells at a low level in the cytoplasm under homeostatic conditions through binding to Kelch-like ECH-associated protein 1 (KEAP1). Nrf2 is released in response to stress signals sensed by KEAP1, translocates to the nucleus, accumulates, and binds to antioxidant response elements (AREs) of target gene promoters (27). Finally, heme oxygenase-1 (HO-1), and glutathione S-transferase (GST) are transcribed, and reactive oxygen species (ROS) removal systems are initiated to protect cells from oxidative stress-induced damages and maintain redox homeostasis (28). Besides, there is increasing evidence that quite different metabolic characteristics and inflammation phenotypes between M1 and M2 macrophages are highly dependent on Nrf2. The negative regulation of Nrf2-related signalings for other transcription factors, such as nuclear factor-κB (NF-κB), may shed light on the link between defense against oxidative stress and reducing inflammation through Nrf2 signaling (24, 29). For example, cyclooxygenase-2 (COX-2), and hypoxia-inducible factor-1α (HIF-1α), which are closely related to the M1 phenotype, could be suppressed *via* the activation of Nrf2 signaling (30). Moreover, the activation of Nrf2 in macrophage could increase the levels of cysteine and glutathione (GSH), by regulating the transporter between cysteine and glutamate, and the GSH-synthesizing enzyme (31, 32). GSH could suppress ROS as a major cellular antioxidant *via* activating HO-1. It has also been reported that the accumulation of GSH could induce an increase in inflammatory factors including NO, IL-1β, IL-4, IL-10, TNF-α, and PGE2, which are related to the M1 phenotype (33). However, in tubular injury resulting from oxidative stress and inflammatory response, the deletion of ROS could promote M2 polarization (34). Similarly, inflammatory response and oxidative stress could be reduced in the cardiac injury induced by LPS, during which process the Nrf2/HO-1 pathway is activated, and the GSH is accumulated (35). Furthermore, it has been reported that the activated Nrf2 could suppress IL-1β without NF-κB or GSH in alveolar macrophages (36). In summary, the relationship is complicated, between the macrophage polarization and metabolic adaptation of macrophages upon inflammatory response and oxidative stress, including OA. As potential mechanisms underlying the protective effects of Nrf2 activation in macrophages, the Nrf2/HO-1 signaling pathway, and the negative regulation of NF-κB signaling will be discussed later.

Furthermore, it has been shown that there is extensive crosstalk between transcriptional pathways involving Nrf2 and NF-κB during oxidative stress and inflammation (29, 32). Generally, Nrf2 signaling negatively regulates NF-κB signaling in oxidative stress and inflammatory response, especially NF-κB (P65) pathway (37). That is, through Nrf2 transcriptional activation, the redox status and metabolism of macrophages

changes, resulting in an anti-inflammatory phenotype (38). Stimulation inducing inflammatory M1 can simultaneously initiate NF-κB-dependent transcriptional pathway inducing the secretion of inflammatory factors quickly, and initiate Nrf2-dependent transcriptional pathway at the same time to cytoprotective response slowly (39). In summary, the action of Nrf2 has a potential role in preventing M1 polarization, and then promoting chondral protection and inhibiting OA progress. For example, in OA, Ca^{2+} influx evoked by transient receptor potential vanilloid 1 (TRPV1) mediated inhibition of M1 macrophage polarization through the phosphorylation of calmodulin-dependent protein kinase II (CaMKII), while the specific inhibitor of Nrf2 counteracted the anti-inflammatory effect (40).

However, the role of Nrf2 in macrophage reprogramming for OA treatment is still unclear to a large extent. Therefore, this review synthesizes evidence of macrophage reprogramming induced Nrf2 inhibition or activation in the progression or treatment of OA.

2 Osteoarthritis pathology driven by macrophages

2.1 M1-induced intra-articular inflammation

The intra-articular microenvironment is characterized by an inflammatory infiltrate largely composed of synovial macrophages. Inflammatory macrophages are believed to be responsible for the presence of OA (23). Damage-associated molecular patterns (DAMPs) are molecules or fragments produced by initial harmful factors that can trigger innate immunity. The DAMPs could activate pattern-recognition receptors for macrophage activation, for example, the toll-like receptor (TLR) 4, and the ligands could be cartilage matrix fragments, or plasma proteins into the articular cavity in OA (41, 42). In contrast, the production of macrophage-derived pro-inflammatory cytokines, such as TNF-α and IL-1β, was greatly reduced by depleting CD14-positive synovial macrophages specifically from OA synovial cells (43).

M1 macrophages tend to secrete pro-inflammatory cytokines including TNF-α and L-1β (44). A series of events triggered by TNF-α and IL-1β can cause cartilage degeneration, where chondrocyte death and cartilage matrix degradation are accelerated while synthesis and regeneration are inhibited (45). Cartilage mainly consists of chondrocytes and extracellular matrix (ECM). Apoptosis of chondrocytes and degeneration of aggrecan (ACAN) and type II collagen (COL2) in ECM are prominent pathological changes in OA (46). It is believed that autocrine TNF-α and IL-1β trigger the pro-inflammatory events in chondrocytes and the catabolic cascades in fibroblast synoviocytes through NF-κB signaling, resulting in the production of IL-6, NO, and prostaglandin E2 (PGE2) (3, 47). The release of IL-6 from macrophages induced by IL-1β can stimulate STAT3 signaling in macrophages, enhancing inflammation responses (48). As a result of IL-6 stimulation, chondrocytes and synovial fibroblasts produce PGE2 and collagenase (49). The high level of NO can inhibit the synthesis of ECM and enhance the activity of matrix metalloproteases (MMPs) (45). By degrading collagen and digesting matrix proteins, MMP1, 3 and 13 can result in skeletal cartilage absorption. The metabolic product of activated COX is arachidonic acid, the substrate of PGE2 biosynthesis (50). PGE2 can also stimulate the release of IL-6 through activating NF-κB pathways (49).

The increase of expression of one of the specific receptor of TNF-α, called TNF receptor I (TNFRI or p55), has been found on OA chondrocytes and synovial fibroblasts (47). In fact, TNF-α plays a central role in the intra-inflammation cascades of OA. TNF-α can break down the cartilage by inhibiting the synthesis of proteoglycan and COL2, and also promoting the apoptosis of chondrocytes. The death domain (DD) in the TNF receptor superfamily is a cytosolic domain and a cysteine-rich extracellular domain. The extrinsic apoptosis pathway is governed by TNF-α, which binds to and interacts with DD, acting on downstream caspases, ultimately leading to apoptosis (45). Similarly, the expression of the specific receptors of IL-1β, called IL-1 receptor type I (IL-1RI), has been found to be increased in human chondrocytes and synovial fibroblasts affected by OA (47). Furthermore, NO can decrease the level of IL-1RA (the antagonist of IL-1R) leading to an increase in IL-1 production (51). IL-1β can also induce apoptosis of chondrocytes relying on endogenous NO in reverse (45). In synovial fibroblasts, IL-1β activates NF-κB (P65) and promotes transcription of IL-6 and PGE2 (52). In chondrocytes, IL-1β can up-regulate the expression of IL-6 through phosphorylating STAT1 and STAT3 (45).

MMPs are a superfamily of proteases that can remodel and degrade ECM in connective tissues. By stimulating the release of MMP1, MMP3, and MMP13 in chondrocytes, TNF-α and IL-1β can affect the synthesis of proteoglycans, connexins, and type II collagen (47). MMP1 and MMP13 are collagenases, while MMP3 is matrix lyases. Apart from that, ADAMTS-4 and ADAMTS-5 belong to the disintegrin and metalloproteinase with thrombospondin motifs family (ADAMTS), which destroys ECM independently from MMPs (53). In chondrocytes, TNF-α and IL-1β can induce an increase in the release of ADAMTS-4 and ADAMTS-5 (45, 54). In addition, direct evidence shows the M1 inflammatory secretion due to interferon-γand TNF-α inhibits chondrogenic differentiation and cartilage repair by up-regulating IL-1β, IL-6, NO, MMP13, and ADAMTS5, and down-regulating ACAN and COL2 (55). To sum up, DAMPs-induced activation of M1 macrophages mainly dependent on NF-κB signaling mediates intra-articular inflammation and cartilage degeneration in OA.

2.2 M2-induced intra-articular anti-inflammation response

The failure in the appropriate proportion of M1 and M2 phenotypes can be the main cause of OA-related low-grade inflammation (56). After all, M2 macrophages are anti-inflammatory and help to repair cartilage in contrast to M1 macrophages. In particular, M2 macrophages can secrete anti-inflammatory factors including IL-10, IL-1RA, chemokine (CeC motif) ligand (CCL18), and pro-chondrogenic mediators including transforming growth factor β (TGF-β) and insulin-like growth factor (IGF) (57). M2 phenotype can be activated by IL-4, IL-10, IL-13, TGF-β, and CCL18, which lead to a positive feedback loop to resolute inflammation (58, 59). Besides, M2 can also regulate collagen turnover pathways in cartilage to promote collagen remodeling (60). The secretion of M2 type can promote cartilage repair by up-regulating COL2 and glycosaminoglycan, inhibiting MMP13, and inhibiting apoptosis of chondrocytes (57).

The previous evidence indicates that IL-10 can protect and repair cartilage, and contributes to the regenerative microenvironment. In patients with OA, IL-10 reduces the specific receptors for TNF-α, and the effects of TNF-α on the fibroblasts by down-regulating PEG2, COX-2, and PLA2 (61). By modulating mitochondrial apoptotic pathways, IL-10 can also inhibit chondrocyte apoptosis by reducing caspase activity and the Bax/Bcl-2 ratio (62). Besides, IL-10 can promote the repairing of chondrocytes and ECM. After IL-10 is administered to compressed articular cartilage *in vitro*, the cell death of chondrocytes, the release of glycosaminoglycans, NO, and molecules that promote ECM degradation and inhibit its syntheses, such as MMP3, MMP13, ADAMTS-4, and inducible nitric oxide synthase (iNOS) are significantly reduced. Moreover, the subsequent study confirmed that the ECM protective effects of IL-10 can be time-dependent (63). Moreover, the overexpression of IL-10 can antagonize the characteristics of cartilage catabolism (MMP3 and MMP13) and the down-regulation of COL2 gene expression induced by TNF-α (64). In addition, the conditioned medium of M1 macrophages decreased the expression of COL2 and ACAN genes in mesenchymal stem cells (MSCs), which are genes associated with chondrogenic differentiation, while M2 macrophages did not exhibit similar inhibition (65).

Furthermore, the increase in IL-10 and IL-1RA can be induced by IL-4, causing the M2 activation in macrophages. The increased IL-10 and IL-1RA, as an anti-inflammatory response, also contribute to responding to the transcription of TNF-α induced by IFN-γ in macrophages. However, in OA synovial fluid, the expression of both is inhibited (66). IL-1RA can be produced by chondrocytes, monocytes, and fibroblasts, belonging to the IL-1 family, and competes with IL-1β to combine with IL-1R type I and II, without triggering the IL-1β-relative downstream inflammatory responses. Although, the production of endogenous IL-1RA needs to be 10-1000 times that of IL-1β to effectively block the binding of IL-1β (67). Additionally, CCL18 is a T-cell chemokine subset associated with the Th2 adaptive responses to IL-4, IL-10, and IL-13. CCL18 has since been identified as a mediator secreted by M2 and an introducer of M2 type (68). Besides, CCL18 can also stimulate fibroblast proliferation and collagen production independent of TGF-β (69). The role of CCL18 in OA is not extremely beneficial, because it has been seen that the relative higher CCL18 levels in synovial fluid of knee OA patients with more serious pathological structural changes (70). Besides, CCL18 can induce the significant enhancement of MMP-3 in fibroblast-like synoviocytes (71).

IGF-1 is a small polypeptide (~7 kDa) belonging to the growth factor family, of which the structure is related to insulin by 50% sequence homology (72, 73). IGF-1 can inhibit chondrocyte apoptosis induced by IL- 1β *in vitro* and can reduce synovitis in OA models (74). Furthermore, IGF-1 inhibits the degradation of cartilage ECM by down-regulating MMP-1, MMP-3, IL-1, and TNF-α (75). Notably, IGF-1 can also play a key role in cartilage anabolism by promoting COL2 and ECM proteoglycan synthesis and decreasing MMP13 to protect the cartilage (76). Through NF-κB signaling, IGF-1 can inhibit the pro-catabolism effects of IL-1β on cartilage, inhibit the apoptosis of chondrocytes (marked by caspase-3), and suppress inflammation (77–79). Moreover, it has been observed that IGF-1 promotes chondrogenic differentiation of adipose-derived MSCs through the expression of COL2, ACAN, and SOX9 (80). IGF-1 can positively regulate chondrogenesis in bone marrow-derived MSCs, and the chondroinductive effects of IGF-1 are independent of TGF-β1 (81). Furthermore, the combination of TGF and IGF can promote chondrogenesis in fracture models *in vivo* (82).

TGF-β family of growth factors and cytokines plays a critical role in skeletogenesis, and can be divided into two major subfamilies, the TGF-β/Activin/Nodal family and the bone morphogenetic protein (BMP) family (83, 84). Signaling by TGF-β1, 2, and 3 is mediated by membrane-bound receptor complexes, which are activated by SMAD proteins intracellularly. Activated type I receptor (also known as ALK5) phosphorylates SMAD2 and SMAD3, which can regulate the expression of target genes related to cartilage anabolism, COL2 for example. However, when TGF-β signals through the ALK1 receptor, phosphorylated SMADs 1/5/8 can be targeted to up-regulate cartilage catabolism genes, like MMP13 (85). The role of TGF-β in OA seems to have two sides, and the administration of TGF-β is equally controversial because the choice between phosphorylation of SMAD2/3 or SMAD5/8 has an unknown mechanism (86).

The conversion of the phenotype of macrophages in OA could play a role in treatment because the secreted cytokines regulate inflammation and cartilage metabolism (Figure 1).

3 Signaling pathways for reprogramming macrophages

The polarization of macrophages dynamically adapts to changes in the microenvironment, and macrophage reprogramming has a complex mechanism. The most studied several pathways relative to reprogramming of macrophages include TGF-β/SMAD, TLR/NF-κB, and JAK/STAT signaling pathways, with the regulation or cooperation of Notch, NLRP3, PI3K/Akt, and MAPK signaling (Figure 2).

3.1 TGF-β/SMAD signaling

Collagen, fibronectins, and fibrinoproteins can form complexes with TGF-β family proteins in the ECM (87). Under the effects of MMPs and/or serine proteases (for example, cathepsins), TGF-β proteins are released from the complex of ECM (86). TGF-β intracellular signaling is mediated by SMAD family members after interaction with two membrane receptors (sequentially phosphorylated type II and type I receptors) (88, 89). Receptor-regulated SMADs (R-SMADs) consisting of SMAD2 and SMAD3 are activated *via* the phosphorylated type I receptor. As a result of the heteromeric trimer with SMAD4, activated R-SMADs can translocate to the nucleus and trigger transcription of target genes (90), including more than 100 transcriptional/signaling regulators, immune modulators, and atherosclerosis-related genes. Dexamethasone-induced M2 polarization is enhanced by TGF-β/SMAD signaling, as type II receptors are elevated, which increases TGF-β response in macrophages. Several genes are associated with the M2 phenotype, including ID3, RGS1, ALOX5AP, TREM1, IL-17RB, JUNB, ELK3, RUNX3, ELL2, TLE3, BCOR, and FOS, and these genes encode functional molecules that are involved in immune responses, inhibiting apoptosis, and maintaining terminal differentiation (91).

In addition, the negative regulators of signal transduction (such as SMAD6 and SMAD7) are also the target genes of TGF-β, which regulates cell homeostasis (92). Of note, A number of E3 ubiquitin ligases, for example, the SMAD ubiquitin regulatory factors and the deubiquitinating enzymes, play a crucial role in recognition and degradation of R-SMADs, SMAD6, SMAD7, and TGF-β receptors. E3 ubiquitin ligases can induce proteasomal degradation *via* the catalysis of their substrates and self-ubiquitination (93, 94). In contrast, deubiquitinating enzymes can antagonize the ubiquitination of E3 ubiquitin ligases, for example USP15 (95). The TGF-β pathway has been studied in OA since it has the potential to regulate cartilage anabolism (86).

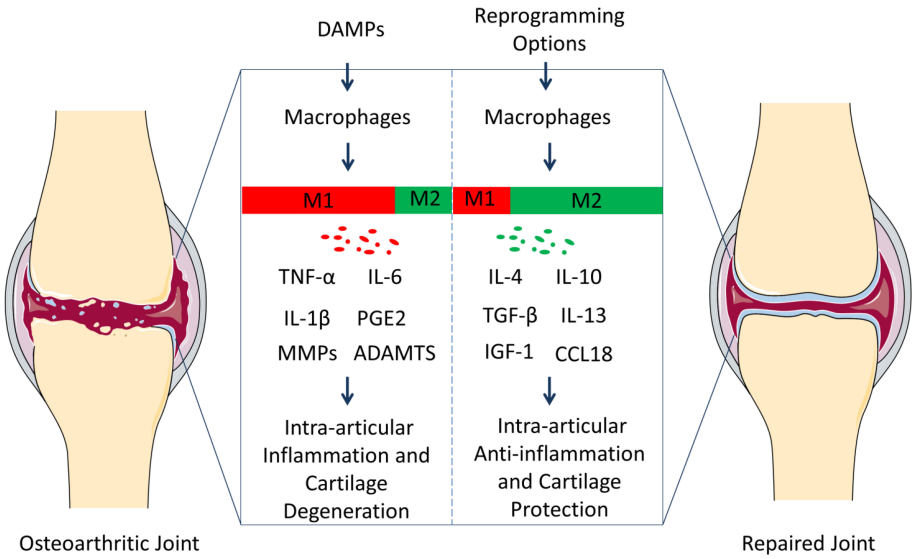

FIGURE 1
Polarization of macrophages in knee osteoarthritis (OA) pathology and repair. Resident macrophages in the synovium have two general phenotypes, M1 and M2. Macrophages tend to serve as M1 phenotype under the inflammation responses caused by damage-associated molecular patterns (DAMPs). M1 subgroups can contribute to OA progression through releasing inflammatory and degenerative molecules including TNF-α, IL-6, IL-1β, PGE2, MMPs, ADAMTS, which leads to synovitis, the death of chondrocytes, and the degradation of extracellular matrix (ECM). However, macrophages can be reprogrammed, and the M2 population can contribute to OA treatment *via* up-regulating anti-inflammatory and regenerative molecules (IL-4, IL-10, TGF-β, IL-13, IGF-1, and CCL18). And the microenvironment can be constructed, where the tissues in the osteoarthritic joints can repair. We suggest that reprograming macrophage phenotypes can be therapeutic targets for the prevention and treatment of OA.

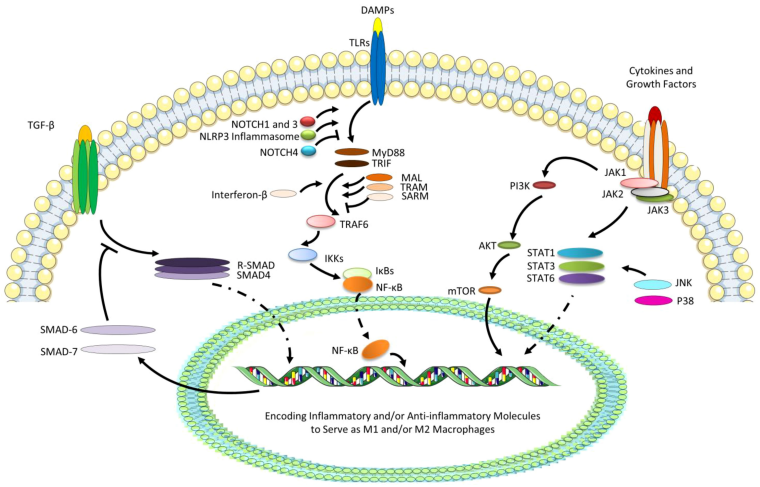

FIGURE 2
The most studied several pathways relative to reprogramming of macrophages. TGF-βcan interact with phosphorylated type II and type I receptors activating receptor-regulated SMADs (R-SMADs, a heterodimer of SMAD2 and SMAD3) to form the heteromeric trimer with SMAD4. The nuclear translocation of R-SMAD can promote the M2 polarization, while SMAD6 and SMAD7 as the negative regulators of TGF-β signaling transduction are also the target genes. Besides, due to the ligation of DAMPs with TLRs, especially TLR4, MyD88 and TRIF, TLR adaptors containing a Toll/IL-1 receptor (TIR) domain, can bind directly to TLRs and recruit MAL and TRAM, while SARM negatively can regulate the pathways. TIR-domain-containing adaptor-inducing interferon-β (dependent on TRIF) can be recruited and then activates TRAF6, and IκBs (especially IκBα) can degrade leading to the release of NF-κB (P50/65) and its translocation to the nucleus, which can promote the M1 polarization. TLR signaling cascade can also activate NOTCH and NLRP3 signaling, which can regulate pro-inflammatory responses *via* the regulated transcription of NF-κB. However, NOTCH4 can negatively regulate the TLR/NF-κB signaling. Finally, STAT/JAK signaling pathway start from tyrosine kinase-associated receptors binding various cytokines and growth factors, followed by the phosphorylation of these receptors and JAKs, and the initiation of the phosphorylation and activation of STATs. Generally, the activation of STAT6 signaling can promote M2 phenotype, while STAT1 and STAT3 can perform complicatedly under the effects of various cytokines or growth factors. JAKs can also activate PI3K/AKT signaling pathway, promoting M2 polarization, which can play a collaborative role in JAK/STAT6 signaling pathway. In addition, STATs can also be activated by JNK and P38, which belongs to extracellular signal-regulated kinases (ERKs), promoting M1 polarization.

3.2 TLR/NF-κB signaling

TLRs are prototype pattern-recognition receptors (PRRs) that recognize pathogen-associated molecular patterns (PAMPs) from microorganisms or DAMPs from damaged tissue (96). TLRs are highly expressed on immune cells, including monocytes, macrophages, and dendritic cells, and can also be up-regulated in response to IL-1 or TLR-4 stimulation in other cells. Through ligation of TLRs, endogenous molecules produced during OA, such as glycoprotein, fibronectin, and hyaluronan of ECM components, have been implicated in activating immune responses (97). Also, plasma proteins can be recognized as DAMPs, such as fibrinogen, which signals through TLR4 to induce inflammatory cytokines (42). There is strong evidence that synovial macrophages and chondrocytes express TLR2 and TLR4, while TLR4 senses more DAMPs than TLR2 in both OA and rheumatoid arthritis (RA). TLR4 plays a key role in the DAMP recognition and the signaling promotion, in the forms of homodimerization or heterodimerization (for example TLR4-TLR6 heterodimers), and the co-receptors, or accessory molecules (such as CD14 and CD36) (41).

There are five TLR adaptors containing a Toll/IL-1 receptor (TIR) domain, among which MyD88 and TRIF bind directly to TLRs and recruit MAL and TRAM, respectively, while SARM negatively regulates these pathways. When the MyD88-dependent TLR4 signaling pathway mediated by TRAM is activated, TIR-domain-containing adaptor-inducing interferon-β (dependent on TRIF) can be recruited and then activates a cascade of proteins including TRAF6, which finally induces the degradation of IκBs, and the release of NF-κB (P50/65) and its translocation to the nucleus (98). NF-κB is central to all macrophage TLR-medicated inflammation responses. IκBs inhibit NF-κB in the cytoplasm by forming complexes with NF-κB. Of note, the regulation of TLR/NF-κB signaling is mainly induced by the ubiquitination/deubiquitination of TRAF6 (99). Macrophages tend to show M1 polarization secreting TNF-α, IL-1β, IL-8, and COX-2. It has been seen that the block of TLR4/NF-κB signaling pathway can inhibit M1 polarization (100–102).

TLR signaling cascade can also activate NOTCH signaling, which regulates pro-inflammatory responses *via* the regulated transcription of NF-κB (103). The Notch gene family encodes evolutionarily highly conserved, single-pass, type I transmembrane heterodimers of 300 kDa called NOTCH receptors (NOTCH1-4 in mammals) which control macrophage activation and polarization *via* TLRs (104). It has

been shown that macrophages up-regulate NOTCH1 upon the activation of TLRs, which implies that NOTCH1 mainly contributes to pro-inflammatory activation (105). In particular, signaling transduction enhances transcriptional activity by phosphorylating and degrading IkBα (106). Similarly, the activation of NOTCH3 by delta-like 4 (Dll4) in macrophages leads to enhanced responses to LPS or IL-1β *via* TLR4/NF-κB pathway (107). Furthermore, NOTCH1 and NOTCH3 cooperate to control the expression of NF-κB-dependent pro-inflammatory genes following TLR-4 activation, with NOTCH3 dominating for the first hours and NOTCH1 later (104). By contrast, the inhibition of NOTCH1 can induce a decrease in M1 polarization and an increase in M2 polarization (108). However, the activation of TLRs/NF-κB pathway in macrophages is negatively regulated by NOTCH4, depending on the phosphorylation of STAT3 and the weakness of STAT1, which activates STAT3/JAK2 signaling (109). Taken together, the regulation of Notch signaling has roles with two sides in TLR/NF-κB pathway in macrophages.

Furthermore, the NLRP3 inflammasome can be activated by DAMPs/TLRs/NF-κB pathway. NLRP3 consists of microparticles, ATP, cholesterol, and microbial toxins, acting as a key sensor of tissue damage and activating sterile inflammation (110). NLRP3 interacts with adapter apoptosis-associated speck-like protein (ASC), and pro-caspase-1 is recruited as an effector, resulting in the formation of NLRP3 inflammasome in the cytosol (111). Different from NOTCH, the role of NLRP3 signaling seems to be pro-inflammation as its effects on processing interleukin precursors (such as pro-IL-1β and pro-IL-18) into mature and secreted interleukin forms (112).

Although the TLR4/NF-κB pathway in chondrocytes has been emphasized as it induces cartilage catabolism (41, 113), the signaling pathway in macrophages has also been targeted. Specifically, chondroitin sulphate inhibits NF-κB and IL-1β secretion from macrophages through inhibition of TLR4 and DAMP interactions (114). On the contrary, lumican, a glycoprotein in adult articular cartilage, has been shown to be up-regulated in OA, and induce the inflammation related to macrophages *via* TLR4 pathway (115).

3.3 JAK/STAT signaling

Signal transducer and activator of transcriptions (STATs) including STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B and STAT6 are latent in cytoplasm, and can be phosphorylated and activated *via* tyrosine phosphorylation and dimerization, finally leading to translocation to the nucleus, binding to promoter sequences and the activation of transcription. The transformation of STATs from latent to active is dependent on Janus kinases (JAKs), which belong to the family of tyrosine kinases (TYKs). JAK1, JAK2, JAK3, and TYK2 have been indicated as JAKs, which can act as tyrosine kinase and bind tyrosine kinase-associated receptors intracellularly. Tyrosine kinase-associated receptors can bind various cytokines and growth factors, followed by the phosphorylation of these receptors and JAKs, and the initiation of the phosphorylation and activation of STATs (116–118).

In macrophages, IL-13 and IL-4 can activate the M2 phenotype by activating JAK2/STAT3 signaling, where IL-4 phosphorylates STAT3 and STAT6, as well as up-regulating DNA binding activity of STAT3, and IL-13 initiates Tyk2 to cascade STAT1 and STAT6, and also to increase DNA binding activity of STAT1 (119). Moreover, STAT6 effects have been widely elucidated in M2 polarization induced by IL-4 and IL-13 (120, 121). IL-10 can activate STAT3 *via* JAK1 and Tyk2 (122). However, LPS and IL-6 can activate the M1 phenotype through JAK2/STAT3 signaling (123, 124). Besides, IFN-γ has an important role in phosphorylation and dimerization of STAT1, leading to M1 phenotype of macrophages (122, 125). Furthermore, STAT1 and STAT3 play antagonistic roles in pro- and anti-inflammation response in macrophages (126), whereas they can also cross-regulate each other in some immune responses, like the additional activation of STAT3 along with the pro-inflammation activation of STAT1 induced by IFN-γ (127).

Besides STATs, JAKs activate the phosphatidylinositol 3-kinase (PI3K) signaling pathway, for example, GM-CSF-induced activation of JAK2 (128). PI3Ks are lipid-signaling kinases that phosphorylate phosphoinositides to form PIP3, 4, and 5 (129, 130). After PI3K activation, 3-phosphoinositide-dependent kinase (PDK1) is recruited and activated. PDK1 phosphorylates and activates protein kinase B (AKT) (131). PI3K activation inhibits macrophage programming into M1, while AKT activation is a critical condition for M2 polarization (132). LPS induces the M1 phenotype in AKT1 ablation, while LPS induces the M2 phenotype in Akt2 ablation (133). The mechanistic target of rapamycin (mTOR) is an evolutionarily conserved PI3K family member, and contributes to the core of the downstream target signaling complexes of PI3K/AKT pathway, called mTORC1 and mTORC2 (130). In LPS-activated M1 polarization, activation of mTORC1 has been demonstrated. Furthermore, mTORC1-mediated feedback inhibition of mTORC2 activity in Akt signaling leads to inhibition of M2 polarization (134, 135). In addition, specific destruction of PI3K/AKT signaling pathway has little effect on JAK/STAT6 signaling, indicating a collaborative role for PI3K/AKT signaling pathway (136).

STATs can also be activated by serine threonine kinases other than JAKs, such as extracellular signal-regulated kinase (ERK) (137). ERK belongs to mitogen-activated protein kinase (MAPK) modules, which also include c-Jun N-terminal kinase (JNK) and P38 (also known as stress-activated protein kinases) (138), and mediate the protein kinase cascades (139). MAPK activation results in nuclear translocation of a number of

transcription factors, including activator protein-1 (AP-1), activating transcription factor (ATF)-2, cAMP-responsive element binding protein (CBP), and members of the ETS family (140). In macrophages, the activation of TLR4 is induced by the phosphorylation cascade of JNK, P38, and ERK, leading to the transcription of NF-κB and AP-1, the increase in the expression of TNF-α and IL-6, forming the activation of M1 polarization (141–143). Among these, pro-inflammatory effects are mainly due to the signaling mediated by JNK and p38 (139). However, the activation of ERK1/2 can inhibit the nuclear translocation of p65 subunit of NF-κB, which results in anti-inflammation response (144, 145). Likewise, the activation of CBP/P300 can mediate the serine phosphorylation of STAT6 induced by IL-4, which can give rise to the M2 phenotype (146, 147).

4 Potential pathways for activating Nrf2 of macrophages in OA as therapeutic choices

Nrf2 belongs to the Cap'n'collar basic leucine zipper transcription factor family, within which the 605 amino acids act their roles as seven highly conserved functional NRF2-ECH homology (Neh) domains (148). It is the Neh2 domain in Nrf2 closest to the N-terminal that binds KEAP1 and is responsible for stabilizing the cytoplasm and degenerating it through ubiquitination. KEAP1 inhibits Nrf2, resulting in stable Nrf2 localization in the cytosol. A homodimer of KEAP1 can also be a stress sensor, recruiting and adapting the E3 ubiquitin ligase cullin-3 (CUL3). In turn, CUL3 can polyubiquitinate Neh2 lysine residues, finally resulting in degradation by ubiquitin proteasomes (149, 150).

Next, from the N-terminal to the C-terminal, there are Neh4, Neh5, Neh7, Neh6, Neh1, and Neh3, respectively. Neh4, Neh5, and Neh3 are transactivation domains that mediate the interaction of Nrf2 with other coactivators. Neh4 and Neh5 can bind CBP/P300 (151, 152), while Neh3 binds chromodomain-helicase-DNA binding 6 (CHD6), contributing to transcription. However, Neh7 and Neh6 are negative regulatory domains for Nrf2, which can bind a β-transducin repeat-containing protein (β-TrCP) and retinoic X receptor α (RXRα), separately (153, 154). The DNA binding domain of Neh1 is mediated by heterodimerization with transcription factors, such as small musculoaponeurotic fibrosarcoma (sMAF) (155).

In cells exposed to stress or electrophilic agents, the alignment of Nrf2 lysine residues is disrupted by the specific thiol residues, leading to the lysine residues being modified by electrophiles within the weak interaction with KEAP1, preventing ubiquitination, and ultimately releasing Nrf2 (156, 157). Nrf2 can translocate to the nucleus after dissociating from KEAP1. With the accumulation of Nrf2 in the nucleus, it forms a heterodimer with sMAF, which binds ARE, a trans-acting DNA enhancer motif. The Nrf2 binding ARE promotes genes encoding cytoprotective proteins, such as GSH-related enzymes, NAD(P)H dehydrogenase quinone 1 (NQO1), and HO-1, to prevent oxidative stress, electrophilic toxicity, and inflammation, and to maintain mitochondrial function and metabolism (158).

Furthermore, polarized macrophages can be used to target synovial inflammation caused by OA (5, 20). There is evidence that Nrf2 activation can inhibit M1 macrophage polarization in OA, which indicates that Nrf2 has a protective role in OA synovitis (40). To a large extent, however, the mechanism linking Nrf2-activation in macrophages remains unknown in the condition of OA. In addition to the existing macrophage polarization mechanism and the treatment strategy of OA, several possible pathways of Nrf2 activation controlling macrophage reprogramming will be discussed (Figure 3).

4.1 Nrf2/HO-1 signaling pathway

Heme oxygenase (HO) is a microsomal enzyme that degrades heme to carbon monoxide (CO), iron, and biliverdin, which plays a protective role in intracellular detoxification during tissue injury. HO regulates a range of anti-inflammatory, antioxidant, and anti-apoptotic pathways through heme degradation, and HO-1 is responsible for most intracellular detoxification among all the HO members (159, 160). The transcription of HO-1 is regulated by the Nrf2/KEAP1/ARE pathway (161), transcription repressor BACH1, AP-1, and several protein kinase signalings (162). Furthermore, HO-1 expression plays a key role in M2 polarization in macrophages (163). For example, activating HO-1 after myocardial infarction can switch M1 macrophages into M2 macrophages (164).

BACH1 plays an important role in Nrf2/HO-1 transcriptional activity (165). BACH1 is a negative regulator of the inducible HO-1 gene expression, that binds sMAF to inhibit HMOX1 transcription by Nrf2, while losing its function in high concentrations of heme (166). BACH1 and Nrf2 competed with each other to regulate ARE-mediated gene expression (167). BACH1-deficient peritoneal macrophages express HO-1 and Arginase-1, Fizz-1, Ym1, and MRC1, which are M2 macrophage markers (168). Nrf2/KEAP1-BACH1 equilibrium has been identified in pulmonary emphysema patients, whereby high levels of BACH1 and KEAP1 result in reduced stress response, mediated by MAPKs, including JNK and ERK (169, 170). The evidence indicates that HO-1 expression mediated by Nrf2 can be regulated strictly by KACH1 in macrophages, and several signaling cascades can control the balance between both, for example MAPKs (171).

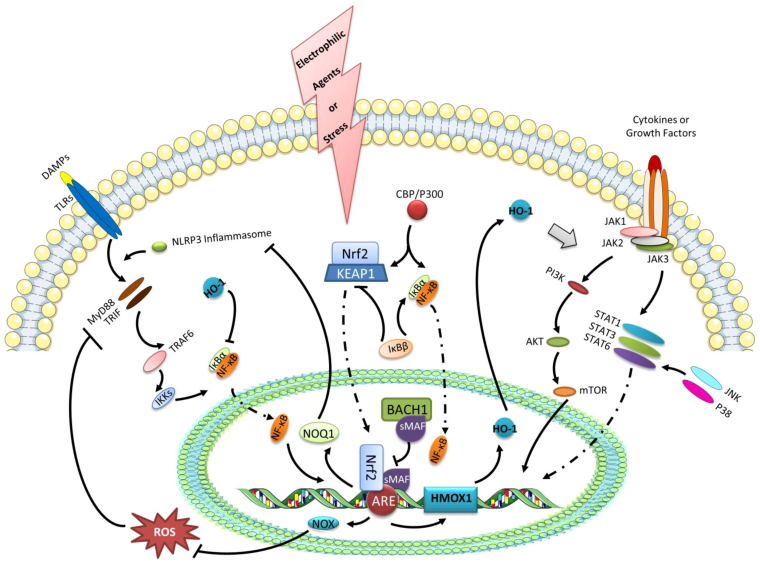

FIGURE 3
The expression of HO-1 and the negative regulation of NF-κB transcription as the possible pathways of Nrf2 activation controlling macrophage reprogramming. Under the stress or electrophilic agents, Nrf2 can translocate to the nucleus after dissociating from KEAP1. With the accumulation of Nrf2 in the nucleus, it forms a heterodimer with sMAF, which binds ARE, a trans-acting DNA enhancer motif. The Nrf2 binding ARE promotes genes encoding cytoprotective proteins, such as GSH-related enzymes, NAD(P)H dehydrogenase quinone 1 (NQO1), and HO-1. BACH1 is a negative regulator of the inducible HO-1 gene expression, that binds sMAF to inhibit HMOX1 transcription by Nrf2.HO-1 can regulate the polarization of macrophages *via* the STATs signaling pathway. Besides, HO-1 also has negative effects on the transcription of NF-κB. Furthermore, the up-regulated NOX1/2 and NOQ1 by Nrf2 activation can suppress NF-κB transcription *via* eliminating ROS and inhibiting NLRP3 inflammasome, respectively. In addition, IκBβ can stabilize KEAP1, while IκBβ can activate IκBα. Likewise, the competitive or antagonistic relationships also exist in the binding of IκBα and KEAP1 to the common activator CBP/P300.

The promoter regions of HO-1 genes contain many AP-1 functional sites (163). AP-1 is made up of heterodimers of members of c-Fos and c-Jun (172). Classical AP-1 is located in the consensus sequence of ARE, and the region of ARE is also the main site for Nrf2 to interact with the HMOX1 promoter (173). A growing body of evidence suggests that inhibition of activated AP-1 helps induce HO-1 expression through Nrf2 (174, 175), which is induced by inhibiting c-Fos (176). It is not clear exactly what role AP-1 plays in Nrf-2 activity, but after all, AP-1's interaction with c-Jun can activate the transcription of GSH-related enzymes and NQO1 (177).

Anti-inflammatory responses in monocytes/macrophages are mediated by an increase in HO-1. Activation of STAT3 and p38/PI3K signaling is necessary to induce HO-1 expression by LPS and IL-10 in rodents (178). LPS-induced activation of HO-1 in M1 phenotypes induces the production of IL-10, as well as the down-regulation of COX-2, iNOS, TNF-α, and IL-6 (179, 180). Inhibition of M1 phenotype with down-regulation of TNF-α induced by globular adiponectin is dependent on IL-10/STAT3/HO-1 pathway in Kupffer cells (181). However, the increase in IL-10 and the decrease in HO-1 have been observed in macrophages stimulated by LPS in humans (182). M1 polarization can be inhibited by HO-1, although this dichotomy suggests that HO-1 controls IL-10 expression in a complex way. Additionally, HO-1 is known to promote M2 phenotype *via* anti-inflammation response and cell protection. HO-1 expression induced by sphingosine-1-phosphate in the supernatant derived from apoptotic cells has been indicated to play a vital role in M2 polarization of macrophages, *via* the STAT1/STAT3 heterodimer (183). Full-length adiponectin induces an M2 phenotype *via* IL-4/STAT6/HO-1, with a decrease in macrophage sensitivity to stimulation by TLR4 ligands, and an increase in M2 markers (184).

4.2 Nrf2 and NF-κB signaling

Transcription factor NF-κB has a central role in inflammation, responsible for the promotion of pro-inflammatory mediators and cytokines (281). Nrf2 and NF-κB signaling regulate the redox homeostasis and inflammation responses, that is, the activation of Nrf2 has been identified to functionally couple the inhibition of NF-κB transcriptional activity (29, 185). For example, the up-regulated HO-1 is also a mediator induced by Nrf2 activation with negative regulatory effects on NF-κB (186). On the one hand, HO-1 can inhibit the degeneration of IκB-α leading to the stabilization of NF-κB in cytoplasm (187). On the other hand, CO induced by HO-1 can

inhibit the TLR/NF-κB signaling *via* binding downstream transcription factor IRF3 and interfering in the downstream pathway (188). Besides, NF-κB family consists of two types, among which p65 has RelA, RelB, and c-Rel transactivation domains, while p50 and p52 do not have transactivation domains. And for transcription, P50 and P52 need to form heterodimerization with the Rel proteins (189). Notably, novel treatments for inflammatory diseases can inhibit NF-κB and activate Nrf2 to alleviate inflammation (190, 191).

The highly electronegative oxygen can accept electrons generated by normal oxidative metabolism within cells, and ROS are produced, which include superoxide anion (O2 -.), hydrogen peroxide (H_2O_2), hydroxyl radical, and singlet oxygen. ROS generated in cytoplasm and mitochondria act their key roles as signaling molecules to regulate physiological processes of macrophages. The ROS generated in cytoplasm and mitochondria serve as important signaling molecules to regulate macrophage physiological processes. NADPH oxidases (NOXs) transfer one electron from NADPH to oxygen to produce cytosolic ROS. Besides, the production of mitochondrial ROS is higher under the biochemical activities of ETC, monoamine oxidases, and P66shc (192). Macrophages could undergo pro-inflammatory cycles promoted by ROS. The targets of ROS include ROS/p38/NF-κB signaling (193), and ROS/p38/STAT1 axis (194), leading to M1 characteristics. And ROS can exacerbate inflammatory response related to NF-κB (p65) (195). However, the role of ROS has two sides. After all, it has been reported that ROS plays a critical role in M2 polarization, during which process the ROS produced by NOX1/NOX2 could contribute to monocyte-to-macrophage differentiation *via* activating of ERK and JNK (196).

Additionally, the activation of Nrf2 can regulate the antioxidant response by controlling the expression of detoxifying enzymes to buffer ROS. Thereby, the decreased ROS results in the inhibition of the activation of NF-κB mediated by oxidative stress (193, 197). Additionally, removing ROS also inhibits STAT1 phosphorylation and conversely activates STAT6 (198, 199). By reducing ROS levels within cells, Nrf2 inhibits oxidative stress-mediated activation, leading to an anti-inflammatory M2 phenotype. Additionally, given the NLRP3 inflammasome signaling as another NF-κB activator, Nrf2 can negatively regulate NLRP3 inflammasome activation (200, 201), especially in the regulative process of ROS. Similarly, Nrf2 can increase NQO1 production and lead to negative effects on NLRP3 inflammasome activation (200). In addition, Hippo-yes-associated protein signaling can also be viewed as a mechanism of Nrf2 activation alleviating inflammation related to NLRP3 inflammasome activation (202).

The mechanisms at a molecular level underlying Nrf2 inhibiting NF-κB are complex. On the one hand, Iκ-B-α is the main inhibitor of NF-κB, which can be phosphorylated by IkB kinase (IKK) β resulting in the release of NF-κB in the pro-inflammation micro-environment. IKKβ can bind KEAP1 *via* the ETGE motif to mediate the ubiquitin and proteasome degradation. KEAP1 is the main inhibitor of Nrf2. When IKKβ is stabilized, KEAP1 can be inhibited, while Iκ-B-α can be phosphorylated, which results in the inhibition of Nrf2 and the activation of NF-κB (203–205). On the other hand, the p65 subunit of NF-κB has been indicated as a partner of KEAP1, and the interaction between the both can inhibit Nrf2-ARE pathway (206). NF-κB (p65) also exerts negative effects on Nrf2 signal transduction by competing for binding with CBP/P300 between p65 and Nrf2. CBP/p300 is not only a co-activator of NF-κB and Nrf2, but also can negatively regulate the biological activity of ARE in the process of transcription under the action of p65 (207, 208). Conversely, the interaction between p65 and KEAP1, and the role of CBP have also been reported as the mechanisms by which Nrf2 negatively regulates NF-κB (209, 210). As a result, the Nrf2 activation can inhibit the production of pro-inflammatory cytokines induced by NF-κB (39, 211). The activated Nrf2 can decrease the release of IL-6 and IL-1β from macrophages by blocking pro-inflammatory cytokine transcription in macrophages (211). Furthermore, M2 polarization induced by the activation of Nrf2 has been reported to treat OA (212).

4.3 Potential treatment strategies for OA linking Nrf2 activation

4.3.1 Itaconate and fumarate derivatives

Fumarate is a mitochondrial metabolite acting as the terminal electron acceptor in the ETC of mammalians (213). In the Krebs cycle, succinate dehydrogenase (SDH) catalyzes the oxidation of succinate to fumarate (214). SDH-induced succinate oxidation can switch the production of ROS and the activation of M1 macrophages (215). And then, the isocitrate dehydrogenase in the Krebs cycle is blocked, inhibiting the canalization of cis-aconitate to isocitrate (216). The accumulated cis-aconitate binding to immune-responsive gene 1 can generate itaconate *via* decarboxylation (214, 217). Itaconate is an endogenous metabolite produced during the TCA cycle, which activates Nrf2 *via* the alkylation of KEAP1 to influence macrophage function (218).

4-octyl itaconate (OI) is an esterified itaconate derivative that can be converted into itaconate by a direct modification of intracellular cysteines (219, 220). Dimethyl fumarate (DMF) is an esterified fumarate derivative, which can be rapidly metabolized into monomethylfumarate (MMF) *in vivo* (220, 221). In macrophages, OI and DMF inhibit pro-IL-1β and NLRP3 signaling pathways activated by TLR4 binding DAMPs (222). However, different from OI, the promoting effect of DMF on KEAP1 alkylation and Nrf2 nuclear accumulation comes from its metabolite MMF (223).

OI and DMF have protective effects on the cartilage in OA. More specifically, OI-induced transcription of Nrf2 in

chondrocytes results in the high expression of HO-1, NQO1, and GCLC, and the low secretion of IL-6, IL-10, MCP-1, and TNF-α, which can switch the prevention from cell death and apoptosis of chondrocytes *via* decreasing oxidative stress and inflammation responses, and put a brake on the progress of OA *in vivo* (224, 225). Similarly, dimethyl fumarate (DMF) can suppress the production of MMP-1, MMP-3, and MMP-13 and the destruction of COL2 induced by TNF-α in OA, which appears to work by inhibiting JAK/STAT3 signaling (226). Moreover, recent evidence shows that exogenous itaconate promotes polarization of M2 macrophages and reduces apoptosis in chondrocytes, a process in which Nrf2 activation-induced stimulator of interferon genes (STING) suppresses transcription of NF-κB (212).

4.3.2 Curcumin

Curcumin is a yellow-colored lipid-soluble polyphenol that is the main active ingredient extracted from the rhizome of Curcuma longa (also known as turmeric) (227). Curcumin has been shown to reduce inflammation and alleviate oxidative stress, where Nrf2 plays a vital role (228). By increasing Nrf2 transcription and HO-1 synthesis, curcumin protects cells from ROS-induced damage and reduces COX-2 production (229, 230). Of note, macrophage COX-2 is an inflammatory enzyme catalyzing the formation of prostaglandins and thromboxane, which is upregulated during pro-inflammatory conditions (231).

The effects of curcumin in OA can also be independent of the Nrf2/HO-1 axis in reducing inflammation, preventing ECM degradation, and promoting cartilage synthesis (232, 233). The effects on inflammation can be induced by suppressing the expression of pro-inflammatory mediators, such as TNF-α, IL-1β, IL-6, IL-17and TGF-β (233–236), which are mainly regulated by NF-κB signaling. Besides, the degeneration of IκBα and the expression of COX-2 in macrophages are reduced by curcumin, resulting in the reduced transcription of NF-κB lower responses to LPS and M1 polarization (236), implying the activation of Nrf2. Curcumin can upregulate the expression of COL2 and downregulate MMP1, MMP3, and MMP13 in ECM protection (235, 237). In addition, the higher level of mRNA related to cartilage anabolism, including COL2A1 and ACAN, can characterize the effect of curcumin on cartilage regeneration (232).

4.3.3 Quercetin

Similar to curcumin, quercetin is one of the most studied and abundant flavonoids found in tea, vegetables, and fruits (238). Quercetin has a high antioxidant activity due to its capacity to up-regulate the transcription of Nrf2, *via* promoting the degeneration of KEAP1 (239). Quercetin can induce the conversion of macrophages from M1 to M2, characterized by lower levels of iNOS-positive cells and inflammatory mediators, and higher levels of CD206-positive cells in diabetic wound healing (240). By scavenging ROS, quercetin-loaded ceria nanocomposite can also increase the M2/M1 ratio of macrophage polarization in periodontal inflammation models (241). Besides, quercetin has been suggested to suppress the expression of iNOS and COX-2, as well as the secretion of IL-6, TNF-α, and IL-1β of M1 macrophages with the decrease in ROS and chemokines related to M1 polarization, including CCL2 and CXCL10, while IL-10 of M2 macrophages has been found up-regulated, with the increase in HO-1 and NQO1 *via* AMPK and AKT signaling pathways (242). Notably, potential mechanisms underlying the effects of quercetin in the immunoregulation of macrophages can also involve the inhibitive effects on the activity of NLRP3 inflammasome *via* TLR2/Myd88/NF-κB and ROS/AMPK pathway (243).

In OA, the effects of quercetin on anti-inflammation response and cartilage protection have been addressed (244). Quercetin can act its anti-inflammatory role in suppressing NO, TNF-α, and IL-1β through inhibiting the NLRP3 signaling pathway, p38 activation, and endoplasmic reticulum stress (245–247). In addition, the activated SIRT1/AMPK signaling pathway can not only suppress the apoptosis of chondrocytes related to endoplasmic reticulum stress, but can also mediate the reversion of mitochondrial dysfunctions and the elimination of ROS in chondrocytes (246, 248). Along aside with anti-inflammation response, the inhibition of cartilage degeneration and the promotion of cartilage regeneration can be characterized by the down-regulation of MMP3, MMP9, MMP13, and ADAMTS-5, while the up-regulation of COL2 (247, 249). Of note, it has been implied that the effects of quercetin can be better loaded by Nano-materials (249, 250). Furthermore, the synovial level of TGF-β1 and TGF-β2 has been found up-regulated after the administration of quercetin due to the increase in M2 macrophages, which can also promote the production of IGF and build a microenvironment promoting chondrogenesis (248).

4.3.4 Melatonin

N-acetyl-5-methoxytryptamine, also known as Melatonin (Mel), is an indolamine with numerous functions in neural, endocrine, and immune physiological activities, playing a versatile role in the regulation of circadian rhythms, the defense against oxidative and inflammation, and the modulation of mitochondrial homeostasis. Mel is synthesized from tryptophan under 5-hydroxytryptamine in multiple extrapineal tissues (251, 252). In OA, Mel has been considered a novel treatment for OA due to its effects on the protection of chondrocytes from apoptosis, the promotion of anabolic metabolism, and the suppression of catabolic metabolism in cartilage, the restoration of redox balance, and the regulation of sirtuin signaling pathways (253).

The role of Mel in the immunoregulation of macrophages has not been verified, however, several studies support this idea. In macrophages, the administration of Mel can induce the activation of Nrf2 and the increased expression of HO-1, while the expression of iNOS and COX-2, and the production of TNF-α, IL-1β and IL-6 can be reduced through the inhibition of NF-κB transcription (254, 255). Besides, the reduced activation of STAT1 and the increased STAT3 can drive the transformation from M1 to M2 phenotype (256). Five potential mechanisms underlying shaping polarization of macrophages have been implied by the review, including through cellular pathways of JAK/STAT, cellular metabolism, miRNAs, mitochondrial dynamics, and mitophagy (257). Furthermore, the most recent study indicates that the interaction of Mel and MT_1 receptors can activate PI3K/Akt and ERK signaling pathways in synovial fibroblasts leading to the up-regulation of microRNA-185a, which can reverse OA-induced pathology in animal models through reducing the secretion of TNF-α, IL-8, and vascular endothelial growth factors (258).

4.3.5 Mesenchymal stem cells

Mesenchymal stem cells (MSCs) are heterogeneous stromal cells commonly sourced from adipose tissue, bone marrow and umbilical cord blood. MSCs have the capacity of differentiating into adipocytes, chondrocytes, and osteoblasts. Except for the capacity of multidirectional differentiation, MSCs exert broad immunoregulatory abilities, which can induce the specific polarization (M1 to M2) of macrophages to promote the repair of damaged tissues *via* cell-to-cell contact and paracrine actions (259–262). The mechanisms and evidence of MSCs mediating the alterations of macrophage phenotypes have been reviewed. Furthermore, the Hippo pathway activating Yes-associated proteins was pointed out as a new mechanism of inhibiting NLRP3 signaling underlying MSCs regulating macrophages (263).

In OA, several studies have revealed the potential effects of MSCs on regulating intra-articular inflammation *via* M2 macrophage polarization. MSCs derived from bone marrow, which are labeled by iron oxide nanoparticles, can induce the increase in CD206-positive cells out of F4/80-positive macrophages, while can also induce the decrease in iNOS-positive cells out of F4/80-positive macrophages in animals experiencing OA due to destabilization of medical meniscus (264). Besides, the cell-free fat extract as a derivative of adipose-derived stem cells, which is rich in cytokines and nutrients, has been reported to be dose-dependently effective in relieving pain (tested by behavioral tests of rats), protecting cartilage, and increasing the ratio of M2 phenotype in the synovium (CD206-positive macrophages) in rat models with sodium iodoacetate-induced OA. The same study also revealed that the cell-free fat can decrease the ratio of CD86-positive cells, and reduce iNOS and COX-2 induced by LPS and IFN-γ in Raw 264.7. And then IL-6 and ADAMTs-5 were reduced, while the expression of SOX-9 was promoted in chondrocytes administrated by the cell-free fat. In addition, ROS can be regulated in these processes (265).

Notably, specifically pre-conditioned MSCs seem to show better regulation of macrophages. MSCs-derived extracellular vesicles (EVs) with antioxidative characteristics *via* over-expressing Nrf2 in adipose MSCs have effects on anti-inflammation and antioxidation. These EVs can induce increased levels of M2 macrophages, and decreased IL-6 and TNF-α (266). According to the International Society for Extracellular Vesicles, EVs are lipid bilayer membrane particles naturally released by the cells. EVs contain proteins, lipids, and nucleic acids, while without a functional nucleus (267). For MSCs, the production of EVs contributes to regulating the activation states of macrophages. The main active substances that play a regulatory role are miRNA and mitochondria transferred through EVs. miRNAs could target various transcription factors (for example, NF-κB) and adaptor proteins (for example, IL-1β) at the post-transcriptional level. Besides, the mitochondrial transfer could be related to the promotion of oxidative phosphorylation and the repair of oxidative stress function (268, 269). The acute lung injury has been ameliorated by MSC-derived small EVs (MSC-EVs) *via* activating Nrf2, during which process the increase in immune and redox mediators, including TLR4, Arg1, and HO-1 could be revealed (270). Besides, in a recent study, nanoparticles simulating EVs are effective for OA by promoting the polarization from M1 to M2. The structure of these EVs is oxidative stress-responsive bilirubin grafted polylysine biomaterial vesicles containing immunoglobulin IgG and berberine (271). However, there is still a knowledge gap in MSC-EVs regulating macrophage polarization *via* Nrf2 in OA treatment. Besides, the expression of Nrf2 in MSCs was promoted by hypoxic preconditioning, while the expression of NF-κB was reduced. And the intrarenal transplantation of these hypoxic preconditioned MSCs was more effective in the activation of HIF-1α/VEGF/Nrf2 signaling to reduce glomerular apoptosis, autophagy, and inflammation (272). However, the evidence of the Nrf2 activation in macrophage has been clearly pointed out in another study. Bone marrow-derived MSCs pre-conditioned by FNDC5, a transmembrane protein acting a crucial role in inflammation diseases, have been found to produce more exosomes. These pre-conditioned exosomes have shown the effects of promoting M2 macrophages and anti-inflammation response in myocardial infarction *via* the inhibition of NF-κB signaling pathway and the activation of Nrf2/HO-1 axis (273).

4.3.6 Low-intensity pulsed ultrasound

Low-intensity pulsed ultrasound (LIPUS) outputs in a pulse wave mode of ultrasound with a non-thermal effect, at an

intensity lower than 3 W/cm^2 (274). The use of LIPUS in OA treatment has been reported as an effective manner in protecting cartilage from degeneration *via* reducing the expression of MMP3, MMP13, and TGF-β1 (275). LIPUS has been considered an effective strategy for patients with OA, which was verified by a recent randomized clinical trial (276).

Besides, LIPUS can promote the cartilage differentiation of bone marrow-derived MSCs due to promoting the nuclear localization of SOX9 dependent on the phosphorylation of ERK1/2 (277). The combination of nanoparticles and LIPUS has been implied to have better effects on OA through inhibiting the degeneration of cartilage (278, 279). Of note, the effects of LIPUS on immunoregulation in macrophages have been reviewed as a potential mechanism of treating OA (20), however, there was little direct evidence. A most recent study has revealed that LIPUS can significantly suppress the secretion of IL-1β, IL-6, and TNF-α induced by LPS in macrophages derived from bone marrow, which can be attributed to increasing the level of intracellular itaconate and the expression of Nrf2 (280).

5 Concluding remarks

In this review, we reviewed the role of macrophage phenotypes in driving and relieving inflammation due to OA and summarized how NF-κB, Nrf2, and their crosstalk shape macrophage polarization. As Nrf2 signaling is believed to impact cellular metabolism, studying the effects of activators of Nrf2 on macrophage metabolism and phenotype related to inflammation could reveal how OA can be treated through reprogramming macrophage functions. Furthermore, studying the effect of Nrf2 activation on macrophages in the OA microenvironment may suggest a potential anti-inflammatory therapy target. Therefore, it is imperative to identify the relationship between Nrf2, macrophage function, and the progression of inflammatory diseases.

Author contributions

Conception and design of study, LW and CH. Drafting of article, LW. Revision of draft, CH. All authors contributed to the article and approved the submitted version.

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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.

Publisher's note

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Cumulative evidence for associations between genetic variants in interleukin 17 family gene and risk of human diseases

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Background: Genetic association studies have elucidated the link of variants in the interleukin 17 (*IL-17*) family genes with susceptibility to human diseases, yet have obtained controversial outcomes. Therefore, we sought to update comprehensive synopsis of variants in the *IL-17* family genes with susceptibility to human diseases.

Methods: Our study screened the Pubmed and Web of Science to enroll eligible articles and performed a meta-analysis, then graded the cumulative evidence of significant association using Venice criteria and false-positive report probability test, and finally assessed the function of variants with strong evidence.

Results: Seven variants in *IL-17* family genes had significant relationships with susceptibility to 18 human diseases identified by meta-analyses. Strong evidence was assigned to 4 variants (*IL-17A* rs2275913, *IL-17A* rs8193037, *IL-17F* rs1889570, *IL-17F* rs763780) with susceptibility to 6 human diseases (lung and cervical cancer, spondyloarthritis, asthma, multiple sclerosis, rheumatoid arthritis), moderate to 2 variants with risk of 5 diseases, weak to 5 variants with risk of 10 diseases. Bioinformatics analysis suggested that the variants with strong evidence might fall in putative functional regions. Additionally, positive relationships for 5 variants with risk of 4 diseases (based on two datasets) and 14 variants with risk of 21 diseases (based on one dataset) were considered noteworthy.

Conclusions: This study offers updated and comprehensive clues that variants in the *IL-17* family genes are significantly linked with susceptibility to cervical, lung cancer, asthma, multiple sclerosis, rheumatoid arthritis and spondyloarthritis, and elucidates the crucial role of the *IL-17* regions in the genetic predisposition to cancer or noncancerous diseases.

KEYWORDS

interleukin 17 family gene, variant, cancer, noncancerous diseases, susceptibility

Introduction

Interleukin (IL) 17 (*IL-17*), a homodimeric glycoprotein composed of 155 amino acids, remains a pro-inflammatory and its family genes contain six groups (*IL-17A* to *F*) (1). The *IL-17* signaling system has a crucial impact on different tissues such as lung, skin, kidney, brain, bone, articular cartilage, meniscus and hematopoietic tissue (1); this system mediated by the binding to *IL-17* receptors can active multiple cell types (such as fibroblasts, endothelial cells, epithelial cells, keratinocytes and macrophages) (2). It could be activated which produces cell subsets of *IL-17* that play a crucial role in multiple essential biological activities and accelerating occurrences of human diseases, involving novel coronavirus disease 2019 (COVID-19) (3).

As early as 2006, Hizawa *et al.* found five single nucleotide polymorphisms (SNPs) in *IL-17F* and found that rs763780 {His-to-Arg substitution at amino acid 161 (H161R)} in the third exon of the *IL-17F* gene influenced the susceptibility to asthma and chronic obstructive pulmonary disease (COPD) in the Japanese population (4, 5). Since then, a range of genetic association studies found that SNPs in *IL-17* family genes have been shown to be linked with the risk of multiple diseases. In 2007, Arisawa *et al.* identified that *IL-17F* rs763780 and *IL-17A* rs2275913 in Japanese population had been shown to be linked with the risk of ulcerative colitis (UC) (6). Subsequently, studies also found that *IL-17* family genes are linked with multiple cancers risk, including ovarian (7), breast (8), hepatocellular (9), esophageal (10), gastric (11) and lung cancer (12). In 2014, two researchers independently performed a meta-analysis and attempted to elucidate the relationship between *IL-17A* rs2275913 and *IL-17F* rs763780 and cancer risk in Asians (13, 14). Interestingly, the outcomes of the two studies were inconsistent. Recently, in an updated meta-analysis conducted in multiple countries from the Asian ancestry, SNP rs2275913 and SNP rs763780 associated with 31,234 subjects were tested. Then it was discovered that *IL-17A* rs2275913 acted as risk factors for gastric, cervical, colorectal and oral cancer, and *IL-17F* rs763780 for cervical and oral cancer (15).

Even though previous studies evaluate the relationship between SNPs in *IL-17* family genes and the risk of diseases, the outcomes are controversial. In addition, an updated research synopsis with comprehensive functional annotation had not been conducted to assess the epidemiological evidence of associations with *IL-17* family genes and risk of all human diseases thus far. Therefore, we carried out meta-analysis to elucidate the relationships of SNPs in the *IL-17* genes with susceptibility to disease, offered the epidemiological evidence for variants with significant relationships, and evaluated the functions of significant variants using public sources.

Materials and methods

Our research followed the guidelines of the Preferred Reporting Items for Systematic Reviews and Meta-Analyses Statement (PRISMA) and the Human Genome Epidemiology Network for systematic review of genetic association studies (16, 17).

We screened genetic association studies from Pubmed and Web of science up to 30 Apr 2022 using "{interleukin-17} OR {IL-17} OR {IL-17}" AND "{variant} OR {variation} OR {polymorphism} OR {genotype} OR {single nucleotide polymorphism} OR {SNP}". We also collected additional articles by retrieving published reviews, meta-analyses studies, etc.

The inclusion criteria are as follows: (i) they were concentrated on the relationships between SNPs in *IL-17* family genes and susceptibility to human cancers or non-neoplastic diseases performed in case-control, cohort or cross-sectional studies (ii) they could provide the genotype data to calculate the odds ratios (ORs) and corresponding 95% confidence intervals (95% CIs) under additive genetic model, (iii) they were published in English by form of full-text. The exclusion criteria are presented, (i) they lacked sufficient information (especially the quantity of genotype and/or allelic distributions), (ii) the study was not focused on SNP in *IL-17* family genes, (iii) they were not published as full reports, such as

conference abstracts and letters to editors, (iv) they concentrated on cancer mortality.

Data extraction

Two authors extracted the data independently using a predesigned collection sheet and any disagreement could be solved with the corresponding author by discussion together. The extracted data were as follows: first author, publishing year, study design, country or region, ethnicity, gene name, variant, cases and controls, genotype counts, minor allelic frequency (MAF). When previous articles studied on the same or overlapping data, we only extracted data from papers with largest sample size and most detailed information.

Statistical analysis

In our study, statistical tests of meta-analysis in the additive genetic association were two-tailed, and a $P < 0.05$ was significant level unless otherwise stated, which were conducted using Stata, version 15 (Stata, College Station, TX, USA). Meta-analyses were performed for variants with at least three datasets. We used the Cochran's Q test to evaluate the heterogeneity between studies (18), while I^2 statistic was applied to quantify and evaluate the heterogeneity (19). Sensitive analyses were performed to evaluate whether the significant association was lost when excluding a single study (dataset), or the first published study, or studies deviated from the Hardy-Weinberg equilibrium (HWE) in the controls. We investigated the probability of an excess of significant findings for single meta-analysis (20). Begg's test and Egger's test were conducted to assess potential publication bias and small-study bias, respectively (21, 22). Moreover, $P<0.1$ as the significant level in the assessment of heterogeneity, an excess of significant findings, Begg's test and Egger's test.

Assessment of epidemiological credibility

Our study graded the epidemiological credibility of significant associations identified by main meta analyses using the Venice guideline (23) and false positive report probability (FPRP) test (24) (see Supplementary Method).

Functional annotation

Our study assessed the potential functional effect of variants on 6p12.2 using data from the Encyclopedia of DNA Elements (ENCODE) tool HaploReg (v4.1) (25) and the UCSC Genome browser (http://genome.ucsc.edu/). We analyzed the regions of promoter or enhancer activity, local histone modification, DNase I hypersensitivity, transcription factor binding motifs and proteins bound to these regulatory sites. In addition, we examined genome-wide cis-eQTL data in multiple tissues from two major eQTL databases: the Genotype-Tissue Expression Project (26) and the Multiple Tissue Human Expression Resource Project (27) to determine whether these genes might explain the observed associations in these loci. We used the data from the Phase 3 of the 1000 Genomes Project to perform linkage disequilibrium (LD) analysis for variants positively associated with susceptibility to cancer and noncancerous diseases in current study (28).

Result

Characteristics of the included studies

As presented in Figure 1, a total of 227 eligible papers including 73,509 cases and 93,253 controls were enrolled (Supplementary Table 1); 135 papers focused on relationships between 10 variants in *IL-17* family genes and 25 diseases (5 cancers as well as 20 noncancerous diseases). The distributions of SNPs (n) with human diseases were presented: asthma (n=5), autoimmune thyroid diseases (AITD) (n=1), cervical cancer (n=3), COPD (n=2), colorectal cancer (n=2), coronary artery disease (CAD) (n=2), functional dyspepsia (FD) (n=3), gastric cancer (n=4), hepatitis B Virus (HBV) infection (n=4), hepatocellular carcinoma (n=1), immune thrombocytopenia (ITP) (n=3), inflammatory bowel disease (IBD) (n=3), leprosy (n=2), lung cancer (n=4), multiple sclerosis (MS) (n=1), osteoarthritis (OA) (n=2), periodontitis (n=2), pre-eclampsia (PE) (n=2), psoriasis (n=1), recurrent miscarriage (RM) (n=1), rheumatoid arthritis (RA) (n=5), spondyloarthritis (SpA) (n=2), systemic lupus erythematosus (SLE) (n=1), tuberculosis (TB) (n=3), Type 1 diabetes mellitus (T1DM) (n=1).

Associations between *IL-17* variants and risk of human diseases

we carried out meta-analyses to investigate correlations of 10 SNPs in *IL-17* family genes with 5 cancers as well as 20 non-cancer diseases based on at least 3 datasets, under an additive genetic model. As presented in Table 1 and Supplementary Table 2, 7 polymorphisms (rs1889570, rs2275913, rs2397084, rs3748067, rs763780, rs8193036, rs8193037) were associated with susceptibility to 4 types of carcinoma (colorectal, cervical, lung and gastric cancer) and 14 non-cancer diseases (AITP, asthma, CAD, HBV infection, ITP, IBD, leprosy, MS, OA, psoriasis, RA, SpA, SLE, TB) (28 associations, $P<0.05$). The cervical cancer susceptibility had positive association with minor allele of rs2275913 in Asians (OR=1.391), rs3748067 (OR=1.493) and

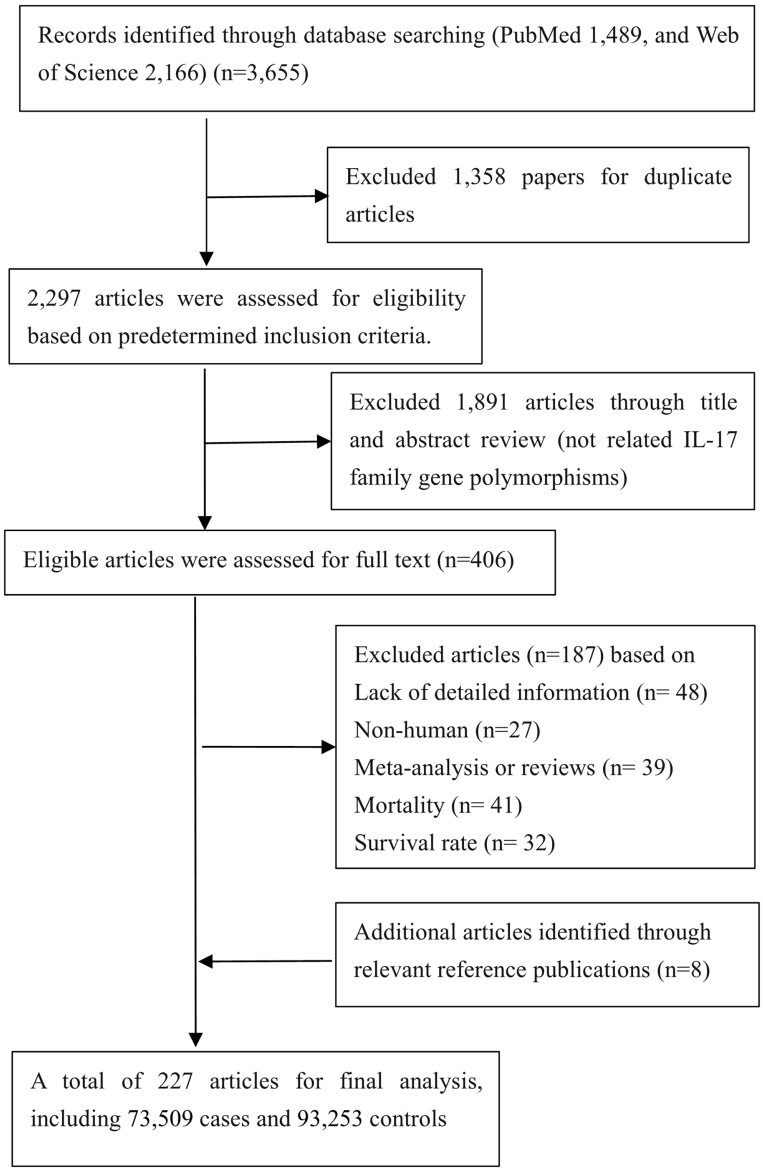

FIGURE 1
Flow diagram of search strategy and study selection.

rs763780 (OR=1.184). Apart from that, rs2275913 had an elevated susceptibility to colorectal cancer (OR=1.452); the positive association could be found in Asians and mixed populations (OR=1.524, OR=2.038, respectively), rather than in Caucasians. Apart from colorectal cancer, rs2275913 had an increased predisposition to gastric cancer (OR=1.281); the positive association could be found both in Asians and Caucasians (OR=1.264, OR=1.723, respectively); this SNP could increase risk of lung cancer (OR=1.213). Moreover, rs8193037 could elevate lung carcinoma susceptibility (OR=2.129).

For non-cancer disease, current results showed that significant relationships with asthma risk were found for rs1889570 in Asians (OR=1.230), rs2397084 (OR=3.220), rs763780 (OR=0.536) and rs8193036 (OR=0.833). In addition, rs763780 had an elevated susceptibility to AITD (OR=1.623) in Asians. For CAD, significant relationships were found for rs2275913 (OR=1.179). Additionally, rs3748067 could reduce risk of CAD in Asians (OR=0.723). For HBV infection, rs763780 had a decreased susceptibility to HBV infection (OR=0.852), especially among Asians (OR=0.826). Apart from that, rs763780 could decrease susceptibility to ITP (OR=0.486) and risk of IBD (OR=0.750) in Asians. Apart from that, rs763780 had a reduced susceptibility to leprosy (OR=0.364) and risk of MS (OR=0.687), respectively.

TABLE 1 Associations between variants in the IL-17 family genes associated with risk of human disease in meta-analysis under additive model (at least 3 datasets).

Gene	Variant	Alleles[a]	Ethnicity	MAF[b]	Number Evaluation			Disease Risk		Heterogeneity		Venice Criteria[c]	FPRP values[d]	Credibility of Evidence[e]
					Studies	Cases	Controls	OR (95%CI)	*P* value	I^2(%)	P_Q			
Asthma														
IL17F	rs1889570	C/T	Overall	0.3826	3	1275	1376	1.109 (0.888-1.384)	0.361	73.5	0.023			
IL17F	rs1889570	C/T	Asian	0.3235	2	856	983	1.230 (1.075-1.401)	0.003	32.9	0.222	ABA	0.032	Strong
IL17F	rs1889570	C/T	Other	0.5305	1	419	393	0.915 (0.753-1.112)	0.371	NA	NA			
IL17A	rs2275913	G/A	Overall	0.3527	6	1051	1741	0.946 (0.690-1.296)	0.728	78.8	< 0.001			
IL17A	rs2275913	G/A	Asian	0.4277	3	774	883	0.976 (0.681-1.400)	0.896	78.7	0.009			
IL17A	rs2275913	G/A	Caucasian	0.2331	3	277	534	0.891 (0.419-1.896)	0.765	85.9	0.001			
IL17F	rs2397084	T/C	Overall	0.0230	3	415	565	3.220 (1.895-5.473)	< 0.001	0.0	0.965	CAA	0.110	Weak
IL17F	rs2397084	T/C	Asian	0.0045	1	221	223	3.572 (0.738-17.292)	0.114	NA	NA			
IL17F	rs2397084	T/C	Caucasian	0.0351	2	194	342	3.167 (1.805-5.556)	< 0.001	0.0	0.826			
IL17F	rs763780	T/C	Overall	0.1060	7	1714	2032	0.925 (0.670-1.277)	0.635	71.6	0.002			
IL17F	rs763780	T/C	Asian	0.1050	5	1520	1690	1.039 (0.743-1.452)	0.824	74.5	0.003			
IL17F	rs763780	T/C	Caucasian	0.0848	2	194	342	0.536 (0.294-0.979)	0.042	0.0	0.363	CAA	0.771	Weak
IL17A	rs8193036	C/T	Asian	0.2856	3	1087	1224	0.833 (0.721-0.962)	0.013	83.1	0.003	ACC	0.197	Weak
Autoimmune thyroid diseases														
IL17F	rs763780	T/C	Asian	0.1161	3	1265	1189	1.623 (1.101-2.393)	0.014	73.8	0.022	BCC	0.444	Weak
Cervical cancer														
IL17A	rs2275913	G/A	Asian	0.3086	6	1634	2235	1.391 (1.263-1.532)	< 0.001	0.0	0.617	AAA	<0.001	Strong
IL17A	rs3748067	C/T	Asian	0.1721	4	1017	1418	1.493 (1.112-2.015)	0.008	69.0	0.022	BCC	0.264	Weak
IL17F	rs763780	T/C	Asian	0.2329	5	1449	1865	1.184 (1.045-1.342)	0.008	7.7	0.363	AAA	0.135	Strong
Chronic obstructive pulmonary disease														
IL17A	rs2275913	G/A	Overall	0.2026	5	1404	1729	1.023 (0.778-1.344)	0.871	76.0	0.002			
IL17A	rs2275913	G/A	Asian	0.4527	1	152	201	0.624 (0.455-0.875)	0.004	NA	NA			
IL17A	rs2275913	G/A	Mixed	0.1695	4	1252	1528	1.163 (0.976-1.385)	0.091	30.1	0.231			
IL17A	rs8193036	C/T	Overall	0.7273	5	1404	1729	1.070 (0.868-1.320)	0.525	65.1	0.022			
IL17A	rs8193036	C/T	Asian	0.2985	1	152	201	1.448 (1.049-2.000)	0.025	NA	NA			
IL17A	rs8193036	C/T	Mixed	0.7833	4	1252	1528	0.995 (0.820-1.208)	0.960	52.5	0.097			
Colorectal cancer														
IL17A	rs2275913	G/A	Overall	0.2714	7	1345	1535	1.452 (1.178-1.790)	< 0.001	65.1	0.009	ACC	0.014	Moderate
IL17A	rs2275913	G/A	Asian	0.3250	3	630	825	1.524 (1.155-2.011)	0.003	63.0	0.067			
IL17A	rs2275913	G/A	Caucasian	0.2120	3	598	610	1.254 (0.871-1.805)	0.223	69.8	0.036			
IL17A	rs2275913	G/A	Mixed	0.1850	1	117	100	2.038 (1.298-3.198)	0.002	NA	NA			
IL17F	rs763780	T/C	Overall	0.1880	6	1841	1307	1.267 (0.933-1.720)	0.130	58.9	0.033			
IL17F	rs763780	T/C	Asian	0.1417	3	1422	867	1.270 (0.825-1.956)	0.278	77.1	0.011			
IL17F	rs763780	T/C	Caucasian	0.0754	2	302	340	1.403 (0.655-3.004)	0.384	65.8	0.087			
IL17F	rs763780	T/C	Mixed	0.9700	1	117	100	1.003 (0.331-3.035)	0.996	NA	NA			
Coronary artery disease														
IL17A	rs2275913	G/A	Overall	0.3293	8	3654	3298	1.179 (1.026-1.354)	0.020	69.4	0.002	ACA	0.272	Weak
IL17A	rs2275913	G/A	Asian	0.3178	6	2534	2411	1.197 (1.053-1.360)	0.006	52.1	0.064			
IL17A	rs2275913	G/A	Caucasian	0.2841	1	220	220	1.527 (1.150-2.026)	0.003	NA	NA			
IL17A	rs2275913	G/A	Mixed	0.1904	1	900	667	0.870 (0.724-1.045)	0.137	NA	NA			
IL17A	rs3748067	C/T	Asian	0.6603	4	1197	1170	0.723 (0.572-0.913)	0.006	55.3	0.082	ACA	0.140	Weak
Functional dyspepsia														
IL17A	rs2275913	G/A	Asian	0.3936	3	175	564	0.815 (0.632-1.052)	0.116	0.0	0.943			
IL17F	rs2397084	T/C	Other	0.0860	3	237	695	0.885 (0.290-2.702)	0.831	79.1	0.008			
IL17F	rs763780	T/C	Overall	0.1178	6	412	1314	0.891 (0.688-1.154)	0.383	0.0	0.667			

(Continued)

TABLE 1 Continued

Gene	Variant	Alleles[a]	Ethnicity	MAF[b]	Number Evaluation			Disease Risk		Heterogeneity		Venice Criteria[c]	FPRP values[d]	Credibility of Evidence[e]
					Studies	Cases	Controls	OR (95%CI)	*P* value	I^2(%)	P_Q			
IL17F	rs763780	T/C	Asian	0.1257	3	175	564	0.676 (0.447-1.022)	0.063	0.0	0.998			
IL17F	rs763780	T/C	Other	0.1120	3	237	750	1.089 (0.779-1.522)	0.617	0.0	0.952			
Gastric cancer														
IL17A	rs2275913	G/A	Overall	0.3845	18	6207	8902	1.281 (1.179-1.392)	< 0.001	60.5	< 0.001	ACA	<0.001	Moderate
IL17A	rs2275913	G/A	Asian	0.3855	17	6046	8731	1.264 (1.165-1.372)	< 0.001	58.4	0.001			
IL17A	rs2275913	G/A	Caucasian	0.3333	1	161	171	1.723 (1.258-2.358)	0.001	NA	NA			
IL17A	rs3748067	C/T	Asian	0.3385	11	3022	5284	0.981 (0.750-1.283)	0.889	83.6	< 0.001			
IL17A	rs4711998	A/G	Asian	0.3316	3	1750	2066	1.001 (0.906-1.105)	0.989	41.7	0.180			
IL17F	rs763780	T/C	Asian	0.6781	10	3944	4566	1.042 (0.805-1.348)	0.756	87.4	< 0.001			
Hepatitis B Virus infection														
IL17A	rs2275913	G/A	Overall	0.4986	13	2633	2479	0.968 (0.834-1.124)	0.671	68.4	< 0.001			
IL17A	rs2275913	G/A	Asian	0.5085	12	2434	2307	0.964 (0.821-1.133)	0.660	70.7	< 0.001			
IL17A	rs2275913	G/A	Caucasian	0.3663	1	199	172	1.024 (0.760-1.381)	0.875	NA	NA			
IL17A	rs4711998	A/G	Asian	0.2324	4	533	617	1.068 (0.678-1.683)	0.776	81.9	0.001			
IL17F	rs763780	T/C	Overall	0.1727	7	1445	1546	0.852 (0.739-0.982)	0.027	82.0	< 0.001	BBC	0.082	Weak
IL17F	rs763780	T/C	Asian	0.1727	6	1246	1374	0.826 (0.713-0.957)	0.011	83.8	< 0.001			
IL17F	rs763780	T/C	Caucasian	0.0610	1	199	172	1.345 (0.760-2.379)	0.309	NA	NA			
IL17A	rs8193036	C/T	Asian	0.2656	6	1260	945	0.947 (0.707-1.269)	0.717	74.7	0.001			
Hepatocellular carcinoma														
IL17A	rs2275913	G/A	Asian	0.5100	4	462	450	1.108 (0.755-1.626)	0.600	72.9	0.011			
Immune thrombocytopenia														
IL17F	rs763780	T/C	Overall	0.2721	6	568	748	0.683 (0.290-1.608)	0.382	89.5	< 0.001			
IL17F	rs763780	T/C	Asian	0.3186	3	413	473	0.486 (0.362-0.653)	< 0.001	0.0	0.966	CAC	0.002	Moderate
IL17F	rs763780	T/C	African	0.1916	3	155	274	0.975 (0.110-8.619)	0.382	94.0	< 0.001			
Inflammatory Bowel Disease														
IL17A	rs2275913	G/A	Asian	0.4202	3	580	764	1.051 (0.783-1.412)	0.740	71.1	0.031			
IL17F	rs763780	T/C	Overall	0.0840	5	1108	2928	0.877 (0.728-1.055)	0.164	51.9	0.081			
IL17F	rs763780	T/C	Asian	0.1531	3	393	994	0.750 (0.585-0.962)	0.024	51.9	0,125	CCC	0.352	Weak
IL17F	rs763780	T/C	Caucasian	0.0486	2	715	1934	1.081 (0.817-1.429)	0.587	0.0	0.417			
IL17A	rs8193036	C/T	Asian	0.2854	3	597	1016	1.012 (0.764-1.340)	0.936	65.1	0.057			
Leprosy														
IL17A	rs2275913	G/A	Mixed	0.2304	3	132	369	0.971 (0.726-1.299)	0.841	6.9	0.342			
IL17F	rs763780	T/C	Overall	0.1791	5	444	537	0.364 (0.268-0.496)	< 0.001	80.8	< 0.001	CCC	<0.001	Moderate
IL17F	rs763780	T/C	Mixed	0.0707	3	304	369	0.870 (0.537-1.409)	0.571	0.0	0.884			
IL17F	rs763780	T/C	Other	0.4167	2	140	168	0.200 (0.131-0.303)	< 0.001	0.0	0.936			
Lung cancer														
IL17F	rs12203582	G/A	Asian	0.5968	4	320	1432	1.103 (0.918-1.325)	0.293	0.0	0.790			
IL17A	rs2275913	G/A	Overall	0.3778	5	559	1690	1.213 (1.039-1.375)	0.014	0.0	0.918	AAA	0.046	Strong
IL17A	rs2275913	G/A	Asian	0.4092	4	320	1432	1.260 (1.053-1.509)	0.012	0.0	0.963			
IL17A	rs2275913	G/A	Caucasian	0.2035	1	239	258	1.088 (0.802-1.477)	0.586	NA	NA			
IL17A	rs3819024	A/G	Asian	0.4426	3	322	1098	0.940 (0.784-1.127)	0.503	0.0	0.832			
IL17A	rs8193037	G/A	Asian	0.1120	3	322	1098	2.129 (1.677-2.702)	< 0.001	49.5	0.138	BBA	<0.001	Strong
Multiple sclerosis														
IL17F	rs763780	T/C	Overall	0.1414	4	774	1064	1.154 (0.608-2.193)	0.661	85.1	< 0.001			
IL17F	rs763780	T/C	Asian	0.1386	2	691	874	0.687 (0.548-0.862)	0.001	0.0	0.884	BAA	0.036	Strong
IL17F	rs763780	T/C	African	0.1737	2	83	190	2.064 (1.348-3.161)	0.001	0.0	0.682			

(Continued)

TABLE 1 Continued

Gene	Variant	Alleles[a]	Ethnicity	MAF[b]	Number Evaluation			Disease Risk		Heterogeneity		Venice Criteria[c]	FPRP values[d]	Credibility of Evidence[e]
					Studies	Cases	Controls	OR (95%CI)	*P* value	I^2(%)	P_Q			
Osteoarthritis														
IL17A	rs2275913	G/A	Overall	0.3448	6	1791	2324	1.232 (1.024-1.483)	0.027	71.7	0.003	ACA	0.347	Weak
IL17A	rs2275913	G/A	Asian	0.3665	3	1306	1383	1.411 (1.105-1.804)	0.006	77.8	0.011			
IL17A	rs2275913	G/A	Caucasian	0.3130	3	485	941	1.048 (0.839-1.310)	0.680	38.8	0.195			
IL17F	rs763780	T/C	Overall	0.1088	7	2671	4420	1.344 (1.111-1.626)	0.002	57.1	0.030	ACA	0.049	Moderate
IL17F	rs763780	T/C	Asian	0.1348	4	2102	3237	1.239 (1.026-1.497)	0.026	57.3	0.071			
IL17F	rs763780	T/C	Caucasian	0.0376	3	569	1138	1.754 (1.157-2.661)	0.008	39.3	0.193			
Periodontitis														
IL17A	rs2275913	G/A	Overall	0.3253	11	935	971	1.304 (0.892-1.908)	0.171	83.0	< 0.001			
IL17A	rs2275913	G/A	Caucasian	0.3547	3	535	524	0.942 (0.782-1.134)	0.526	0.0	0.744			
IL17A	rs2275913	G/A	Mixed	0.2920	4	300	347	1.040 (0.514-2.103)	0.914	84.0	< 0.001			
IL17A	rs2275913	G/A	Other	0.2800	4	100	100	2.498 (0.993-6.235)	0.052	74.3	0.009			
IL17F	rs763780	T/C	Overall	0.0563	5	500	507	1.251 (0.840-1.860)	0.270	0.0	0.520			
IL17F	rs763780	T/C	Mixed	0.0656	4	300	347	1.506 (0.955-2.373)	0.078	0.0	0.935			
IL17F	rs763780	T/C	Caucasian	0.0375	1	200	160	0.658 (0.281-1.543)	0.336	NA	NA			
Pre-eclampsia														
IL17A	rs2275913	G/A	Overall	0.4129	3	1923	2296	1.009 (0.919-1.108)	0.848	0.0	0.860			
IL17A	rs2275913	G/A	Asian	0.4111	2	1662	2018	1.020 (0.922-1.129)	0.702	0.0	0.896			
IL17A	rs2275913	G/A	Caucasian	0.4245	1	261	278	0.950 (0.745-1.210)	0.676	NA	NA			
IL17F	rs763780	T/C	Overall	0.0979	3	1923	2296	0.995 (0.852-1.162)	0.947	54.4	0.111			
IL17F	rs763780	T/C	Asian	0.1017	2	1662	2018	0.938 (0.793-1.109)	0.451	0.0	0.342			
IL17F	rs763780	T/C	Caucasian	0.0737	1	261	278	1.449 (0.948-2.217)	0.087	NA	NA			
Psoriasis														
IL17F	rs763780	T/C	Overall	0.1219	6	1151	975	1.499 (0.899-2.499)	0.121	80.2	< 0.001			
IL17F	rs763780	T/C	Asian	0.0955	2	324	363	1.571 (1.118-2.207)	0.009	0.0	0.371	BAA	0.307	Weak
IL17F	rs763780	T/C	Caucasian	0.0274	2	601	462	1.378 (0.811-2.343)	0.236	0.0	0.371			
IL17F	rs763780	T/C	African	0.0750	1	60	60	4.111 (1.856-9.105)	< 0.001	NA	NA			
IL17F	rs763780	T/C	Other	0.4567	1	166	150	0.676 (0.492-0.929)	0.016	NA	NA			
Recurrent Miscarriage														
IL17A	rs2275913	G/A	Overall	0.4725	3	290	309	1.148 (0.753-1.752)	0.520	65.6	0.055			
IL17A	rs2275913	G/A	Caucasian	0.5265	2	170	189	0.924 (0.666-1.282)	0.634	0.0	0.451			
IL17A	rs2275913	G/A	African	0.3875	1	120	120	1.634 (1.137-2.348)	0.008	NA	NA			
Rheumatoid arthritis														
IL17A	rs2275913	G/A	Overall	0.3661	13	3826	4011	0.862 (0.833-0.955)	0.001	6.8	0.378	AAA	0.001	Strong
IL17A	rs2275913	G/A	Asian	0.4633	1	615	839	0.875 (0.754-1.016)	0.080	NA	NA			
IL17A	rs2275913	G/A	Caucasian	0.3908	8	2452	2389	0.896 (0.824-0.974)	0.010	27.4	0.209			
IL17A	rs2275913	G/A	Mixed	0.2687	4	759	783	0.897 (0.744-1.082)	0.255	5.0	0.368			
IL17F	rs2397084	T/C	Caucasian	0.1056	4	800	695	1.575 (0.499-4.968)	0.439	94.9	< 0.001			
IL17A	rs3819024	A/G	Overall	0.3712	3	2053	2266	0.914 (0.834-1.002)	0.056	0.0	0.603			
IL17A	rs3819024	A/G	Asian	0.4816	1	615	839	0.880 (0.758-1.021)	0.092	NA	NA			
IL17A	rs3819024	A/G	Caucasian	0.3842	1	937	928	0.913 (0.800-1.043)	0.179	NA	NA			
IL17A	rs3819024	A/G	Mixed	0.1513	1	501	499	1.019 (0.798-1.300)	0.882	NA	NA			
IL17F	rs763780	T/C	Overall	0.0713	9	1297	1264	1.650 (0.792-3.437)	0.181	89.9	< 0.001			
IL17F	rs763780	T/C	Caucasian	0.0751	6	1039	980	2.070 (0.794-5.395)	0.137	92.9	< 0.001			
IL17F	rs763780	T/C	Mixed	0.0581	3	258	284	0.977 (0.527-1.812)	0.941	23.4	0.271			
IL17A	rs8193036	C/T	Overall	0.3941	3	2052	2261	1.074 (0.905-1.276)	0.414	65.8	0.054			

(Continued)

TABLE 1 Continued

Gene	Variant	Alleles[a]	Ethnicity	MAF[b]	Number Evaluation			Disease Risk		Heterogeneity		Venice Criteria[c]	FPRP values[d]	Credibility of Evidence[e]
					Studies	Cases	Controls	OR (95%CI)	*P* value	I^2(%)	P_Q			
IL17A	rs8193036	C/T	Asian	0.2555	1	615	839	1.250 (1.056-1.479)	0.010	NA	NA			
IL17A	rs8193036	C/T	Caucasian	0.2974	1	936	923	0.953 (0.827-1.097)	0.502	NA	NA			
IL17A	rs8193036	C/T	Mixed	0.7986	1	501	499	1.050 (0.843-1.309)	0.662	NA	NA			
Spondyloarthritis														
IL17A	rs2275913	G/A	Mixed	0.2500	4	439	784	1.401 (1.156-1.698)	0.001	25.2	0.260	BBA	0.015	Strong
IL17F	rs763780	T/C	Mixed	0.0522	4	439	788	3.684 (2.737-4.960)	< 0.001	0.0	0.431	BAA	<0.001	Strong
Systemic lupus erythematosus														
IL17A	rs2275913	G/A	Overall	0.2338	5	941	1289	1.160 (1.007-1.336)	0.040	53.5	0.072	ACA	0.429	Weak
IL17A	rs2275913	G/A	African	0.2807	3	515	695	1.281 (1.071-1.533)	0.007	63.2	0.066			
IL17A	rs2275913	G/A	Caucasian	0.3105	1	59	95	0.975 (0.592-1.604)	0.920	NA	NA			
IL17A	rs2275913	G/A	Mixed	0.1573	1	367	499	0.985 (0.758-1.281)	0.910	NA	NA			
Tuberculosis														
IL17A	rs2275913	G/A	Overall	0.4066	12	4240	4983	0.998 (0.854-1.165)	0.975	79.5	< 0.001			
IL17A	rs2275913	G/A	Asian	0.4283	5	3137	3549	1.066 (0.894-1.270)	0.478	80.4	< 0.001			
IL17A	rs2275913	G/A	Caucasian	0.4039	5	727	1052	1.120 (0.799-1.572)	0.511	79.4	0.001			
IL17A	rs2275913	G/A	Mixed	0.2287	2	376	382	0.594 (0.399-0.884)	0.010	48.6	0.163	BBA	0.406	Weak
IL17A	rs3748067	C/T	Asian	0.1429	3	2365	2305	1.244 (0.939-1.649)	0.129	71.4	0.030			
IL17F	rs763780	T/C	Overall	0.0990	8	3574	4238	1.380 (1.084-1.758)	0.009	72.0	0.001	ACA	0.188	Weak
IL17F	rs763780	T/C	Asian	0.1139	5	3137	3549	1.426 (1.082-1.879)	0.012	0.0	0.665			
IL17F	rs763780	T/C	Caucasian	0.0225	3	437	689	1.088 (0.607-1.951)	0.776	83.3	< 0.001			
Type 1 diabetes mellitus														
IL17A	rs2275913	G/A	Overall	0.3288	3	155	184	1.223 (0.887-1.685)	0.219	31.4	0.233			
IL17A	rs2275913	G/A	Other	0.2333	2	30	30	0.580 (0.229-1.466)	0.249	0.0	0.782			
IL17A	rs2275913	G/A	Caucasian	0.3474	1	125	154	1.360 (0.965-1.918)	0.079	NA	NA			

OR, odds ratio; A, adenine; T, thymine; G, guanine; C, cytosine; NA, not applicable.
[a]Major alleles (reference)/minor alleles.
[b]Frequency of minor allele in controls.

Interestingly, current results showed that rs2275913 could increase risk of OA (OR=1.232), SpA (OR=1.401) and SLE (OR=1.160), whereas decrease susceptibility to RA (OR=0.862) and TB (OR=0.594); rs763780 could increase risk of OA (OR=1.344), psoriasis (OR=1.571), SpA (OR=3.684) and TB (OR=1.380), respectively.

Additionally, 8 SNPs (rs12203582, rs2275913, rs2397084, rs3748067, rs3819024, rs8193036, rs763780 and rs4711998) had no association with susceptibility to 14 human diseases (asthma, COPD, colorectal cancer, FD, gastric cancer, HBV infection, hepatocellular carcinoma, IBD, lung cancer, PE, RM, RA, TB and T1DM) in additive model. Of these, 3 SNPs had no association with 3 diseases (rs3748067 and rs763780 for gastric cancer; rs2275913 for HBV infection; rs3748067 for TB) with at least 2,300 case and 2,300 controls. Also, we calculated the statistical power to confirm whether the bigger sample size confirming these relationships is required in next study (Table 2 and Supplementary Table 3).

Heterogeneity, bias and sensitivity analysis

As shown in Table 1, heterogeneity was investigated for 28 significant associations (7 SNPs for 18 human diseases). Mild heterogeneity (I^2 < 25%) was assigned to 3 variants with risk of 2 cancers and 6 noncancerous diseases (10 associations); moderate heterogeneity (25% ≤ I^2 ≤ 50%) was assigned to 3 variants with risk of 1 cancer and 3 noncancerous diseases (4 associations); high heterogeneity (I^2 > 50%) was assigned to 4 variants with risk of 3 cancers and 9 noncancerous diseases (14 associations). Moreover, the results indicated that publication bias existed (p < 0.10) in associations for rs3748067 and rs2275913 with colorectal cancer risk. Apart from that, sensitivity analyses indicated that some significant summary ORs were lost, including rs8193036 in asthma and AITD, rs763780 in HBV infection (excess of significant findings); rs763780 in AITD and ITP (small study), and in IBD (HWE), and in leprosy (small study).

TABLE 2 Variants in IL-17 family genes showing no relation to risk of disease in meta-analyses in additive model.

Gene	Variant	Alleles[a]	Disease	Ethnicity	MAF[b]	Number Evaluation		Meta-analysis risk		Heterogeneity	
						Studies	Sample size (case/control)	OR (95%CI)	P_{value}	I^2(%)	$P_{(Q)}$
IL17A	rs2275913	G/A	Asthma	Overall	0.3527	6	2792 (1051/1741)	1.108 (0.755-1.626)	0.600	72.9	0.011
IL17A	rs2275913	G/A	COPD	Overall	0.2026	5	3133 (1404/1729)	1.051 (0.783-1.412)	0.740	71.1	0.031
IL17A	rs8193036	C/T	COPD	Overall	0.7273	5	3133 (1404/1729)	1.012 (0.764-1.340)	0.936	65.1	0.057
IL17F	rs763780	T/C	Colorectal cancer	Overall	0.1880	6	3148 (1841/1307)	1.103 (0.918-1.325)	0.293	0.0	0.790
IL17A	rs2275913	G/A	Functional dyspepsia	Asian	0.3936	3	739 (175/564)	0.940 (0.784-1.127)	0.503	0.0	0.832
IL17F	rs763780	T/C	Functional dyspepsia	Overall	0.1178	6	1726 (412/1314)	1.304 (0.892-1.908)	0.171	83.0	< 0.001
IL17A	rs3748067	C/T	Gastric Cancer	Asian	0.3385	11	8306 (3022/5284)	1.251 (0.840-1.860)	0.270	0.0	0.520
IL17A	rs4711998	A/G	Gastric Cancer	Asian	0.3316	3	3816 (1750/2066)	1.009 (0.919-1.108)	0.848	0.0	0.860
IL17F	rs763780	T/C	Gastric Cancer	Asian	0.6781	10	8510 (3944/4566)	0.995 (0.852-1.162)	0.947	54.4	0.111
IL17A	rs2275913	G/A	Hepatitis B Virus infection	Overall	0.4986	13	5112 (2633/2479)	1.148 (0.753-1.752)	0.520	65.6	0.055
IL17A	rs4711998	A/G	Hepatitis B Virus infection	Asian	0.2324	4	1150 (533/617)	1.575 (0.499-4.968)	0.439	94.9	< 0.001
IL17A	rs8193036	C/T	Hepatitis B Virus infection	Asian	0.2656	6	2205 (1260/945)	1.650 (0.792-3.437)	0.181	89.9	< 0.001
IL17A	rs2275913	G/A	Hepatocellular carcinoma	Asian	0.5100	4	912 (462/450)	1.074 (0.905-1.276)	0.414	65.8	0.054
IL17A	rs2275913	G/A	Inflammatory Bowel Disease	Asian	0.4202	3	1344 (580/764)	1.244 (0.939-1.649)	0.129	71.4	0.030
IL17A	rs8193036	C/T	Inflammatory Bowel Disease	Asian	0.2854	3	1613 (597/1016)	1.223 (0.887-1.685)	0.219	31.4	0.233
IL17F	rs12203582	G/A	Lung cancer	Asian	0.5968	4	1752 (320/1432)	1.108 (0.755-1.626)	0.600	72.9	0.011
IL17A	rs3819024	A/G	Lung cancer	Asian	0.4426	3	1420 (322/1098)	1.051 (0.783-1.412)	0.740	71.1	0.031
IL17A	rs2275913	G/A	Periodontitis	Overall	0.3253	11	1906 (935/971)	1.012 (0.764-1.340)	0.936	65.1	0.057
IL17F	rs763780	T/C	Periodontitis	Overall	0.0563	5	1007 (500/507)	1.103 (0.918-1.325)	0.293	0.0	0.790
IL17A	rs2275913	G/A	Periodontitis	Overall	0.4129	3	4219 (1923/2296)	0.940 (0.784-1.127)	0.503	0.0	0.832
IL17F	rs763780	T/C	Periodontitis	Overall	0.0979	3	4219 (1923/2296)	1.304 (0.892-1.908)	0.171	83.0	< 0.001
IL17A	rs2275913	G/A	Recurrent Miscarriage	Overall	0.4725	3	599 (290/309)	1.251 (0.840-1.860)	0.270	0.0	0.520
IL17F	rs2397084	T/C	Rheumatoid arthritis	Caucasian	0.1056	4	1495 (800/695)	1.009 (0.919-1.108)	0.848	0.0	0.860
IL17F	rs763780	T/C	Rheumatoid arthritis)	Overall	0.0713	9	2561 (1297/1264)	0.995 (0.852-1.162)	0.947	54.4	0.111
IL17A	rs8193036	C/T	Rheumatoid arthritis	Overall	0.3941	3	4313 (2052/4313)	1.148 (0.753-1.752)	0.520	65.6	0.055
IL17A	rs3748067	C/T	Tuberculosis	Asian	0.1429	3	4670 (2365/2305)	1.575 (0.499-4.968)	0.439	94.9	< 0.001
IL17A	rs2275913	G/A	Type 1 diabetes mellitus	Overall	0.3288	3	339 (155/184)	1.650 (0.792-3.437)	0.181	89.9	< 0.001

OR, odds ratio; A, adenine; C, cytosine; G, guanine; T, thymine;
[a]Major alleles (reference)/Minor alleles.
[b]Frequency of minor allele in controls.

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Cumulative evidence of association

As shown in Table 1, our study firstly used the Venice guideline and FPRP tests to grade epidemiological credibility of 28 significant. In terms of Venice guideline, strong, moderate and weak evidence were assigned to 4, 7 and 17 associations, respectively. Then, the probability for a true association between the 28 positive associations was investigated based on FPRP tests. The FPRP value < 0.05 was observed for 13 associations, FPRP 0.05 to 0.2 for 6 associations, and FPRP > 0.2 was found for 9 associations, respectively. At last, combing Venice guideline and FPRP tests, strong evidence was assigned to 4 variants (*IL-17F* rs1889570, *IL-17A* rs2275913, *IL-17F* rs763780, *IL-17A* rs8193037) and 2 cancer (cervical and lung cancer) as well as 4 noncancerous diseases (asthma, MS, RA, SpA), moderate to 2 SNPs (*IL-17A* rs2275913, *IL-17F* rs763780) and colorectal and gastric cancer as well as 3 noncancerous diseases (ITP, leprosy, OA), weak to 5 SNPs (*IL-17F* rs2397084, *IL-17F* rs763780, *IL-17A* rs8193036, *IL-17A* rs3748067, *IL-17A* rs2275913) and 1 cancer (cervical cancer) as well as 9 noncancerous diseases (asthma, AITD, CAD, HBV infection, IBD, OA, psoriasis, SLE, TB).

In addition, we attempted to pool the ORs and 95%CIs on the basis of two datasets and found that 9 variants (rs1889570, rs2275913, rs3819024, rs4711998, rs4819554, rs6973569, rs763780, rs8193036 and rs8193037) had significantly associated with susceptibility to 4 cancers (bladder, colorectal, papillary thyroid cancer and hepatocellular carcinoma) as well as 10 noncancerous diseases (ankylosing spondylitis, Behcet's disease, bronchiolitis, brucellosis, chronic chagas cardiomyopathy, CAD, gastro-duodenal ulcer, recurrent miscarriage, silicosis and TB); of these, 5 SNPs (rs4819554, rs8193036, rs8193037, rs2275913, rs763780) and risk of 4 noncancerous diseases (ankylosing spondylitis, CAD, gastro-duodenal ulcer, recurrent miscarriage) were considered noteworthy (P < 0.2 for FPRP) (see Supplementary Table 4). Additionally, we calculated the ORs and 95% CI in the additive model to assess the relationships between 53 variants and susceptibility to 90 diseases (based on one dataset), yielding significant relationships between 22 variants and the risk of 47 types of carcinoma. Apart from that, P value of FPRP for the significant associations also be calculated. Finally, we considered the associations between 14 variants and susceptibility to 21 diseases noteworthy (see Supplementary Table 5).

Functional annotation for variants with strong evidence

As shown in Table 3, we used the Encyclopedia of DNA Elements tool HaploReg v4.1 to assess the potential functional roles for strong evidence (4 variants with risk of 6 human diseases). For functional annotations, rs763780 was annotated as missense. The total 4 SNPs might be located in a region with strong promoter and enhancer activity, and two SNPs in alteration in regulatory motif. Subsequently, as the consequence of the function evaluation using the PolyPhen-2 web server (29), the unique non-synonymous variant rs763780 was qualitatively predicted to be "benign" with a naïve Bayes posterior probability of less than 0.15. As shown in Supplementary Figure 1, the linkage disequilibrium (LD) plots presented that the regions represented by significant SNPs had distinct genetic structures among in European, Asian and African ancestry. The information extracted from the Genotype-Tissue Expression Project shows that rs2275913, rs763780, rs8193037, rs1889570 are eQTLs for the *IL-17A, IL-17F, GSTA8P, MCM3*. In addition, rs2275913 had an increased expression in *GSTA8P, IL-17A* genes in testis tissues; rs763780 and rs8193037 had an increased expression in *MCM3,IL-17F* genes in muscle and esophagus tissues, respectively (Supplementary Table 6). In our study, rs2275913 and rs763780 had significantly associated with susceptibility to cervical cancer and SpA. The Phase 3 of the 1000 Genomes Project (30) (Supplementary Table 7) indicated *IL-17A*

TABLE 3 Summary of functional annotations for 4 SNPs in 6 human diseases (strong epidemiological credibility).

Variant	Gene	Position[a]	Annotation	Promoter histone marks[b]	Enhancer histone marks[c]	DNAse[d]	Proteins bound[e]	Motifs changed[f]
rs763780	IL17F	52101739	missense	ESDR, BLD	BLD, HRT			Lmo2-complex, Mtf1
rs1889570	IL17F	52110734		ESDR, IPSC, BRST	GI, LIV			HDAC2,NF-I, NRSF
rs2275913	IL17A	52051033		BLD	BLD			
rs8193037	IL17A	52051109		BLD, GI	BLD			

[a]chromosome position is based on NCBI Build 37.
[b]Histone modification of H3K4me1 and H3K27ac (tissue types: if >3, only the number is included).
[c]Histone modification of H3K4me3 (tissue types: if >3, only the number is included).
[d]Levels of DNase I hypersensitivity (tissue types: if >3, only the number is included).
[e]Alteration in transcription factor binding (disruptions: if >3, only the number is included).
[f]Alteration in regulatory motif (disruptions: if >3, only the number is included).

rs2275913 is uncorrelated with *IL-17F* rs763780 in Europeans, East Asians and Africans (r^2< 0.05 for all tests). Moreover, *IL-17A* rs2275913 and *IL-17A* rs8193037 had associated with predisposition to lung cancer. We also found that rs2275913 is weak LD with rs8193037 in East Asians (r^2 = 0.1) and is uncorrelated with rs8193037 in Europeans and Africans (r^2< 0.05).

Discussion

Our study performs a comprehensive research synopsis and meta-analysis, summarizes and updates the associations between SNPs in *IL-17* family genes and predisposition to human diseases for the first time, which offers precise results for the SNPs and provides more variants and diseases that never been investigated before. Our study included 227 papers with 73,509 cases and 93,253 controls and performed a meta-analysis using 135 papers with available information to assess relationships of 10 variants with susceptibility to 25 diseases (5 cancers as well as 20 non-cancer diseases); 7 SNPs had positively associated with 18 human disease predisposition. Our study used the Venice guidelines and FPRP tests to grade the cumulative evidence of significant relationships. At last, 4 SNPs were assigned to strong evidence with predisposition to 6 human diseases (9 associations: *IL-17F* rs1889570 in asthma; *IL-17A* rs2275913 in lung cancer, cervical cancer, RA, SpA; *IL-17F* rs763780 in cervical cancer, MS, SpA; *IL-17A* rs8193037 in lung cancer), moderate to 2 SNPs and 2 cancer as well as 3 noncancerous diseases, weak to 5 SNPs and 1 cancer as well as 9 noncancerous diseases. Moreover, we attempted to construct functional annotations for these 4 variants with strong evidence using data from the Encyclopedia of DNA Elements Project and other public databases and then uncovered that the SNPs with strong evidence might fall in several putative regulatory regions. In summary, this study provides updated evidence that SNPs in the *IL-17* family genes had significant associations with predisposition to lung, cervical cancer, asthma, RA, SpA and MS.

The *IL-17*, a kind of proinflammatory cytokine, plays a crucial role in both innate and acquired immune responses (31). Previous papers demonstrated that *IL-17* is activated by microbial products, and may accelerate carcinoma occurrence and development by angiogenic functions (32). The *IL-17A* gene (Gene ID: 3605) is located at chromosome 6p12.2, and the encoded protein is a proinflammatory cytokine produced by activated T cells, which might involve in the development of human diseases (31). In the previous paper, it was pointed out that *IL-17A* rs2275913 acted as risk factor for multiple cancers (gastric, cervical, colorectal and oral carcinoma) (15) and non-cancerous diseases (RA) (33). Consistent with our meta-analysis, strong evidence was assigned to *IL-17A* rs2275913 in lung cancer, cervical cancer, RA, SpA, and *IL-17A* rs8193037 in lung cancer. LD analysis indicates that *IL-17A* rs2275913 and *IL-17A* rs8193037 were associated with susceptibility to lung cancer. We also found that rs2275913 is weak LD with rs8193037 in East Asians and is uncorrelated with rs8193037 in Europeans and Africans, indicating that the functional mechanisms of the two variants associated with lung cancer risk may be distinct in different ethnic groups and partly explain why some variants are found to be associated with a cancer site in one ethnic group but not in others. Current evidence presents that high expression of *IL-17A* is linked with the development and progression of cancers, and *IL-17A* could be regulated at the transcriptional level (34). *IL-17A* rs2275913 could influence the expression of the *IL-17A* protein and trigger cell transformation and maintain the autonomous proliferation of the transformed cells, and thus increase the susceptibility of cervical cancer, especially in HPV infection individuals (35). *IL-17A* could influence the transcriptional activity of *NFAT* and trigger the stimulation of T lymphocytes cells, which might increase risk of lung cancer (36). Moreover, a recent study indicated that the G allele polymorphism of *IL-17A* rs2275913 (a change from glutamic acid to lysine) was protective in RA individuals (37), which is consistent with our results; *IL-17A* and *TNF-α* had been considered as a predictor of a poor outcome in RA individuals; interestingly, previous study concluded that therapies targeting *IL-17* in autoimmune diseases ameliorated the inadequate response to anti-*TNF-α* therapy (38), which indicated that SNP rs2275913 could be considered as a novel target for gene therapy of RA and promote drug developments against RA. Moreover, the SNP rs2275913 A allele is linked with high IL-17 expression, which has an elevated susceptibility to autoimmune and inflammatory diseases, including SpA (39). Moreover, previous papers found that drugs target other molecules of the immune system, such as anti-IL-17A (ixekizumab and secukinumab) and anti-IL-17A receptor (brodalumab). The efficacy of anti-IL-17R and anti-IL-17 agents has been shown in Phase II (40) and III trials (41, 42), indicating that IL-17A might have impact on the pathogenesis of psoriasis.

The *IL-17F* gene (Gene ID:112744) is located at chromosome 6p12.2, and the protein encoded by *IL-17F* gene is a cytokine activated by T cells. It could stimulate the production of other cytokines, such as *IL-6*, *IL-8*, and *CSF2/GM_CSF* (31, 43). These cytokines might have similar synergistic effects on risk of human diseases (44). In the previous paper, it was pointed out that *IL-17F* rs763780 might trigger the development of cervical and oral carcinoma (15) and non-cancerous diseases (such as asthma) (45). Consistent with our meta-analysis, strong evidence was assigned to *IL-17F* rs1889570 in asthma, and *IL-17F* rs763780 in cervical cancer, MS, SpA. In our study, rs2275913 and rs763780 had positive association with

susceptibility to SpA and cervical cancer. LD analysis indicates that *IL-17A* rs2275913 is uncorrelated with *IL-17F* rs763780 in Europeans, East Asians and Africans, demonstrating that there might be different causal variants and functional mechanisms involved in relationships of variants in the *IL-17A-IL-17F* genes with risk of cervical cancer and SpA. Current evidence presents that *IL-17F* rs1889570 could increase risk of asthma by influencing the expression of proinflammatory cytokines, chemokines, and growth factors associated with leukocyte activation and airway remodeling (46). Additionally, a previous study found that the *IL-17F* rs763780, a missense located in the *IL-17F* exon3 region, could trigger high *IL-17* expression which influenced cervical cancer cell growth, and thus increased risk of cervical cancer (47). Moreover, the SNP rs763780 C allele is linked with high IL-17 expression, which has proved to increase risk of SpA (39).

Additionally, we calculated the ORs and 95% CI in the additive model to assess the relationships between 53 variants and susceptibility to 90 diseases (based on one dataset), yielding significant relationships between 22 variants and the risk of 47 types of carcinoma. For example, our results found that some non-cancerous diseases including autoimmune diseases (such as autoimmune thyroid diseases), alopecia areata, and some type of autoimmune blistering diseases (such as bullous pemphigoid), which have presented in our supplementary Tables (see Supplementary Table 1). Moreover, in our study, we performed meta-analysis based on at least three datasets. However, we found that alopecia areata and bullous pemphigoid only contained 1 dataset for each SNP, which could not be assessed by meta-analysis. Therefore, we just presented these information in our supplementary files (see Supplementary Table 1 and Supplementary Table 5). Finally, we hope to attempt to collect more information in order to solve this issue in our study in the future. Apart from that, *P* value of FPRP for the significant associations also be calculated. In summary, we considered the associations between 14 variants and susceptibility to 21 diseases noteworthy (see Supplementary Table 5). Further, well-designed studies are recommended to clarify the association with multiple diseases for these variants.

Additionally, 8 SNPs had no association with susceptibility to 14 human diseases in additive model. Of these, 3 SNPs had no association with 3 diseases (rs3748067 and rs763780 for gastric cancer; rs2275913 for HBV infection; rs3748067 for TB) with at least 2,300 cases and 2,300 controls, which presented over 80% statistical power to detect an OR of 1.15 for a variant with MAF 0.20 (Type 1 error 0.05). Further study less than current individuals on these 3 variants for these 3 diseases will not yield fruitful results (Supplementary Table 3). Apart from that, our study identified that significant relationships for 5 variants with risk of 4 diseases (based on two datasets) and 14 variants with risk of 21 diseases (based on one dataset) were considered noteworthy, which might be required to confirm or refute these associations by large-scale studies in the future.

Some limitations apply to this research: (i) even though we conducted a comprehensive search to screen eligible papers, some articles may have been missed. Also, some malignancies and non-cancer diseases could not be completely assessed by meta-analysis owing to insufficient information (for example, lack of genotype amount, fewer than 3 datasets in some associations); (ii) only ethnicity was assessed by subgroup, other factors (such as pathological/clinical type, gene-gene or gene-environment associations and interactions) might be required to confirm or refute the relationships with susceptibility to disease; (iii) the unreasonable data, like errors in genotype, could not be investigated, and (iv) moderate and weak evidence should be explained with caution.

In summary, this large-scale meta-analysis identified that 4 SNPs in the *IL-17* family genes were graded as demonstrating strong association to 2 cancer and 4 non-cancer disease risk. Apart from that, these findings provide a foundation for further demonstrating the variations in the *IL-17* family genes are positively linked with susceptibility to cervical cancer, lung cancer, asthma, MS, RA, SpA, and highlight that the variants in *IL-17* family genes might become a valuable genetic tool to investigate the pharmacological targeting potential of *IL-17* family genes. We should further understand its biological pathway and apply these clues to clinical practice and public health for risk assessment and management.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Author contributions

TL, LY, and HC designed this work. TL and LY integrated and analyzed the data. TL, LY, and HC wrote this manuscript. TL, LY, XL, CZ, CJ, ZY, CF, and HC finished the related Tables and Figures. TL, LY, and HC edited and revised the manuscript. All authors approved this manuscript.

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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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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.1008184/full#supplementary-material

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Ferroptosis and musculoskeletal diseases: "Iron Maiden" cell death may be a promising therapeutic target

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Ferroptosis is a novel form of cell death precisely regulated by iron metabolism, antioxidant processes, and lipid metabolism that plays an irreplaceable role in the development of many diseases. Musculoskeletal disorders (MSKs), including osteoporosis, osteoarthritis, rheumatoid arthritis, intervertebral disc degeneration, sarcopenia, and rhabdomyolysis, have become one of the most common causes of disability and a major burden on public health and social care systems. The mechanism of ferroptosis in MSKs has recently been elucidated. In this review, we briefly introduce the ferroptosis mechanism and illustrate the pathological roles of ferroptosis in MSKs with a focus on how ferroptosis can be exploited as a promising treatment strategy. Notably, because the toxicity of compounds that inhibit or induce ferroptosis in other organs is largely unknown, ferroptosis appears to be a double-edged sword. We point out that more research is needed in the future to verify the therapeutic effects based on ferroptosis in MSKs.

KEYWORDS

regulated cell death, immunology, oxidative stress, inflammation, mechanism

Introduction

Classically, the regulation of cell death is assumed to be achieved by two main models: accidental cell death (ACD) and regulated cell death (RCD) (1). ACD is usually triggered by an unexpected injury or attack, which overwhelms any possible molecular control mechanism (2). Whereas, the process of RCD, manifested as classical apoptosis, is regulated by a number of molecules with genetically defined effector and precise signaling cascades involving unique immunological, functional, and biochemical consequences. A growing body of evidence in recent years reveals that many nonapoptotic forms of RCD,

including pyroptosis, necrosis, autophagy, and ferroptosis, contribute to various pathologies in humans (3).

In 2002, Dolma et al (4). performed a study of the Epithelial cells expressing oncogenic Ras (RasV12) cell line and found that erastin, a novel compound, initiates a cell death process displaying no apoptotic features, such as fragmented nuclei, DNA laddering, and activated caspase 3, which later came to be known as ferroptosis. The cell death model ferroptosis was officially recognized as a novel form of RCD in 2012 (5) (see Figure 1 for a glossary of key terms in ferroptosis). Ferroptosis involves many pathophysiological processes characterized by lipid peroxidation caused by the accumulation of iron-dependent reactive oxygen species (ROS) in cells. The mechanisms and regulatory pathways of ferroptosis are complicated and involve a variety of signaling molecules and metabolic pathways (Figures 2, 3). Of note, ferroptosis participates in the occurrence and development of various diseases.

Aging, a natural and complex physiological process, is generally considered the greatest risk factor for many neurodegenerative, metabolic, cardiovascular, and musculoskeletal disorders (MSKs). Among these conditions, MSKs, including a wide range of inflammatory and degenerative diseases such as osteoporosis, osteoarthritis (OA), rheumatoid arthritis (RA), and sports injuries, are some of the most common causes of chronic disability worldwide (21). With a large number of cases, MSKs remain a disease of international concern, which has resulted in an enormous global disease burden (22). Therefore, therapeutic targets linking aging and disease may extend the healthy life span of patients and limit healthcare costs.

In recent years, neurological, cardiovascular, and neoplastic diseases have been the focus of both ferroptosis research and clinical applications (23–25). As a hot topic, ferroptosis is now known to play a critical role in multiple systems or organs (26). Interestingly, a growing body of evidence has recently uncovered links between ferroptosis and MSKs. However, there are relatively few reviews in the field of MSKs, which gives us an opportunity to remedy this major deficiency. Herein, we summarize the basic pathological features of ferroptosis and discuss its potential role in the pathophysiology of these diseases and associated complications.

Mechanisms and regulation of ferroptosis

The mechanism of ferroptosis has been summarized almost perfectly (27, 28). In general, ferroptosis has its own morphological, biochemical, and genetic characteristics. Morphologically, mitochondrial shrinkage, which involves decreased mitochondrial cristae and increased membrane density, is a characteristic feature of ferroptosis (29). Biochemically, ferroptosis is activated by the formation of iron-dependent ROS, which can be inhibited by antioxidants and iron chelators rather than apoptosis, necrosis, or autophagy inhibitors (5). The broad biological processes include iron metabolism, antioxidant processes, and lipid metabolism.

Iron metabolism

All these observations allowed the identification of iron as a critical cofactor in various biochemical enzyme-catalyzed reactions involved in the physiological regulation of oxygen transport, energy metabolism, DNA synthesis, and repair. Iron

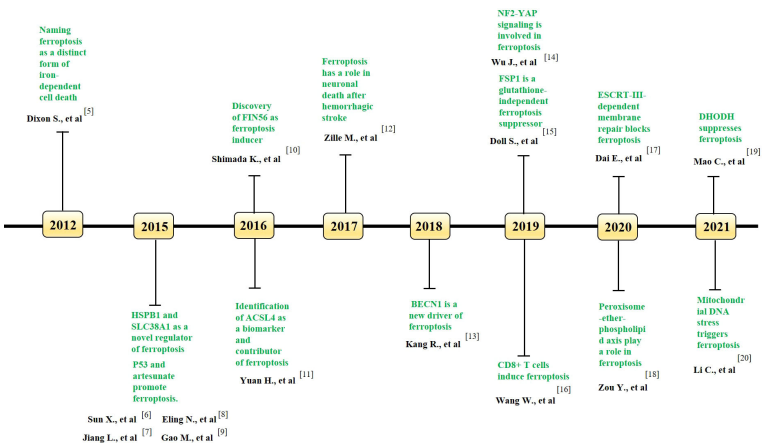

FIGURE 1
Key milestones in the literature of ferroptosis over time. The key discoveries related to ferroptosis in each year is indicated. GPX4, Glutathione peroxidase 4; HSPB1, heat shock protein beta-1; SLC38A1, solute carrier family 38 member 1; FIN56, Ferroptosis inducing 56; ACSL4, acyl-coenzyme A synthetase long-chain family member 4; BECN1, beclin 1; NF2, neurofibromin 2; YAP, Yes-associated protein; FSP1, ferroptosis suppressor protein 1; ESCRT, endosomal sorting complexes required for transport; DHODH, dihydroorotate dehydrogenase.

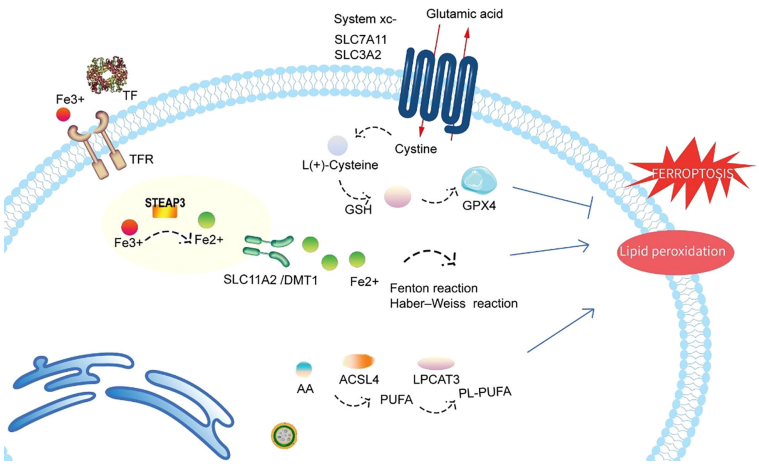

FIGURE 2
Schematic representation of the mechanism of ferroptosis. Ferroptosis is a novel form of cell death precisely regulated by iron metabolism, antioxidant processes, and lipid metabolism. Fe3+ imported through the transferrin receptor is converted to Fe2+ in endosomes and released from endosomes by DMT1 (also known as SLC11A2). Excess Fe2+ can induce ferroptosis through reactive oxygen species generated by the Fenton reaction and Haber–Weiss reaction. GPX4 is the major endogenous mechanism that suppresses lipid peroxidation. Cystine enters cells through system Xc-, and cystine is subsequently converted to cysteine, which generates glutathione (GSH), a cofactor for GPX4. Both LPCAT3 and ACSL4 also exert direct or indirect actions on lipid peroxidation of membrane PUFAs. TF, transferrin; TFR, transferrin receptor; STEAP3, six-transmembrane epithelial antigen of prostate 3; SLC11A2/DMT1, divalent metal transporter 1; SLC7A11, solute carrier family 7 member 11; SLC3A2, solute carrier family 3 member 2; system xc-, cystine/glutamate antiporter system; GPX4, glutathione peroxidase 4; GSH, glutathione; PL-PUFA, phospholipid-bound polyunsaturated fatty acids; PUFA, polyunsaturated fatty acid; ACSL4, acyl-coenzyme A synthetase long-chain family member 4; LPCAT3, lysophosphatidylcholine acyltransferase 3.

has implications in several disorders of phosphate and bone metabolism (30). Hydroxyl radicals (HO·), generated by Fe2+ through the Fenton reaction, might be associated with damage to proteins, lipids, and DNA.

The maintenance of iron homeostasis is crucial for the normal function of cells. Several studies have found that abnormal iron metabolism as a result of iron overload is the main characteristic of ferroptosis. Circulating iron binds to transferrin receptor 1 (TFR1) on the cell membrane, and in this reaction, ferric iron is reduced to ferrous iron by the six-transmembrane epithelial antigen of prostate 3 (STEAP3). Subsequently, divalent iron is released by divalent metal transporter 1 (DMT1) into the labile iron pool (LIP) in the cytoplasm. Of note, lysosomes, which store large quantities of LIP, are considered the main organelles responsible for cellular ferroptosis and represent promising potential disease targets (31). Nuclear receptor coactivator 4 (NCOA4)–mediated ferritinophagy increases the degradation of ferritin by lysosomes, reduces iron storage, and promotes ferroptosis (32). Excess bivalent iron is then transported extracellularly by ferroportin 1 (FPN1) and stored in ferritin heavy chain 1 (FTH1) and ferritin light chain 1 (FTL1). In addition, both treatments with the ferroptosis inhibitor ferrostatin-1 (Fer-1) and hepatocyte-specific knockout of the metal transporter Slc39a14 significantly reduce iron overload-induced liver ferroptosis in transferrin knockout mice (Trf-LKO) mice (33).

From a physiopathological point of view, ferritin is a strong buffer involved in regulating iron deficiency and maintaining homeostasis (34). The regulation of mitochondrial iron metabolism is assumed to be achieved by mitochondrial ferritin, and its overexpression can reverse ferroptosis induced by erastin (35). Under pathological conditions, during the process of Fenton and Haber–Weiss reactions, iron overload induces ferroptosis by producing high concentrations of ROS (36, 37).

Deferoxamine (DFO), an iron chelator, works by inhibiting ferroptosis as a result of intracellular iron overload (38). In addition, mitochondrial transferrin mitoferrin 1/2 is destroyed on the inner mitochondrial membrane, which consequently results in abnormal iron metabolism in the mitochondria (39). In summary, increased iron intake, reduced stable iron, and decreased iron outflow ultimately stimulate oxidative damage and lead to ferroptosis.

Antioxidant systems

Glutathione peroxidase 4

Glutathione peroxidase 4 (GPX4), one of the eight glutathione (GSH) peroxidases, serves as the primary intracellular antioxidant buffer, which plays an indispensable

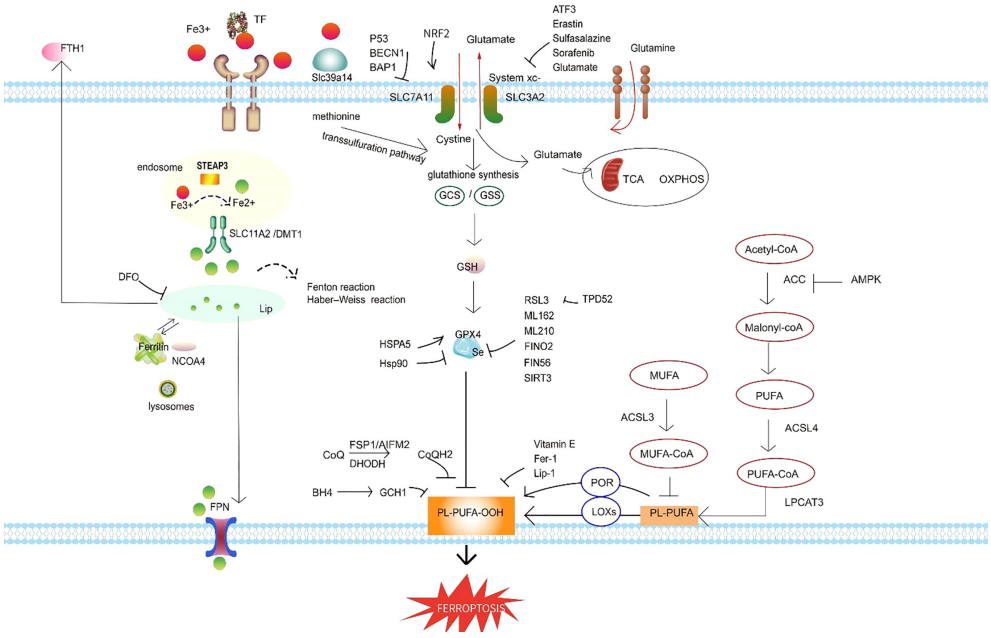

FIGURE 3
Ferroptosis regulatory pathways. The regulatory pathways of ferroptosis can be roughly classified into three types. The first involves iron metabolism, including the regulation of ferritin degradation by NCOA4. The second is the GSH/GPX4 pathway, which includes system xc inhibition, the transsulfuration pathway, and the glutamine pathway. The third is lipid metabolism, including ACSL4, ACSL3, and LPCAT3, which are related to lipid regulation and ferroptosis. In addition, FSP1 inhibits ferroptosis independently of GSH, and the BH4–GCH1 axis effectively inhibits lipid peroxidation and thereby defends against ferroptosis. Abbreviations: TF, transferrin; FTH1, ferritin heavy chain 1; DFO, defetoxamine; LIP, labile iron pool; NCOA4, nuclear receptor coactivator 4; FPN, ferroportin; SLC11A2/DMT1, divalent metal transporter 1; STEAP3, six-transmembrane epithelial antigen of prostate 3; NRF2, NF-E2–related factor 2; SLC7A11, solute carrier family 7 member 11; SLC3A2, solute carrier family 3 member 2; system xc-, the cystine/glutamate antiporter system; Slc39a14, the zinc transporter Zip14; BECN1, beclin 1; BAP1, BRCA-1–associated protein; ATF3, recombinant activating transcription factor 3; GCS, glutamylcysteine synthetase; GSS, recombinant glutathione synthetase; TCA, tricarboxylic acid; OXPHOS, oxidative phosphorylation; GPX4, glutathione peroxidase 4; GSH, glutathione; HSPA5, heat shock protein A5; HSP90, heat shock protein 90; RSL3, RAS-selective-lethal-3; $FINO_2$, an endoperoxide-containing 1,2-dioxolane; FIN56, ferroptosis-inducing 56; SIRT3, sirtuin 3; TPD52, tumor protein D52; Fer-1, ferrostatin-1; Lip-1, liproxstatin-1; FSP1, ferroptosis suppressor protein 1; AIFM2, apoptosis-inducing factor mitochondria-associated 2; BH4, tetrahydrobiopterin; GCH1, GTP cyclohydrolase-1; PL-PUFA-OOH, lipid peroxides; POR, NADPH-cytochrome P450 reductase; LOXs, lipoxygenases; PUFA, polyunsaturated fatty acid; MUFA, monounsaturated fatty acid; PL-PUFA, phospholipid-bound polyunsaturated fatty acids; ACSL4, acyl-coenzyme A synthetase long-chain family member 4; LPCAT3, lysophosphatidylcholine acyltransferase 3; ACC, acetyl CoA carboxylase; AMPK, AMP-activated protein kinase.

role in antioxidant effects and ferroptosis regulation. It has been demonstrated that selenium (Se) can improve GPX4 expression (40). The conversion of reduced GSH to oxidized glutathione (GSSG) is achieved by either the conversion of free hydrogen peroxide to water or the reduction of lipid peroxides (L-OOH) to lipid hydroxyl derivatives (LOH), and both are essential for the maintenance of cellular redox homeostasis (41). In addition, both the GSH/GSSG ratio and GSH reflect the oxidation resistance and are therefore associated with ferroptosis (42).

The overexpression of mitochondrial GPX4 inhibits mitochondrial oxidative stress and mitochondrial-dependent apoptosis, whereas its deficiency leads to massive cell death (43). As a substrate of GPX4, RAS-selective-lethal-3 (RSL3) serves as a ferroptosis-induced molecule that works by binding to GPX4 in an iron-, mitogen-activated protein kinase kinase (MEK)-, and ROS-dependent manner. Genetically enhancing tumor protein D52 (TPD52)–dependent lipid storage prevents RSL3-induced lipid peroxidation and subsequent ferroptosis *in vitro* and *in vivo* (44). However, the overexpression of GPX4 may induce resistance to RSL3. GPX4 activity is also inhibited directly or indirectly by other chemical compounds, such as ML162, ML210, Diphenyleneiodonium chloride (DPI) compounds, buthionine sulfoximine, sirtuin 3, $FINO_2$, and FIN56 (45, 46). In addition, studies have shown that the activation of transcription factor 4 (ATF4) leads to the induction of HSPA5, which, in turn, binds to GPX4 and prevents GPX4 protein degradation and subsequent lipid peroxidation (47). On the basis of the existing scientific research, HSP90 family members may act on GPX4, which

results in inhibition of the antioxidant capacity of GPX4 by inhibiting its activity (48).

Cystine/glutamate antiporter system

The selective inhibition of the cystine/glutamate antiporter system (system xc-) works by decreasing intracellular GSH, exacerbating the accumulation of ROS, and eventually leading to ferroptosis (49). System xc- is composed of solute carrier family 3 member 2 (SLC3A2) and solute carrier family 7 member 11 (SLC7A11), and the major negative effect of system xc- on ferroptosis regulation appears to be due to its crucial role in the synthesis of the antioxidant GSH, which allows the exchange of exogenous cystine with glutamate in a 1:1 ratio. GSH is then synthesized by cysteine, which is degraded from cystine (50, 51). In addition, studies have verified that many exogenous compounds or endogenous genes can activate or inhibit system xc-. Genetically, system xc- could be positively regulated by NF-E2–related factor 2 (NRF2) and ubiquitin thioesterase. In addition, BRCA-1–associated protein (BAP1) and p53 can negatively regulate system xc- (52). The ATF3 enhances the ferroptosis induced by erastin *via* the repression of system xc- (53), whereas AMP-activated protein kinase (AMPK)–mediated beclin 1 (BECN1) phosphorylation increases ferroptosis by directly inhibiting system xc- activity (54). Radiotherapy and immunotherapy enhance lipid oxidation and the ferroptosis of tumor cells by synergistically suppressing SLC7A11 (55). Sorafenib and sulfasalazine inhibit system xc- function and induce ferroptosis (56, 57), whereas GDF15 knockdown facilitates ferroptosis induced by erastin *via* the attenuation of SLC7A11 expression (58). Moreover, P53 can enhance ferroptosis by inhibiting the expression of SLC7A11 (59).

Cysteine availability restricts GSH biosynthesis, whereas cysteine starvation induces GSH depletion and ferroptosis. When the available cysteine is limited, some cells utilize the transsulfuration pathway to transform methionine to cysteine (60). Glutamate is also an important regulator of ferroptosis. At high concentrations, this compound suppresses system xc- and triggers ferroptosis. Glutamine degradation (*via* glutaminolysis) fuels the tricarboxylic acid (TCA) cycle and provides the basis for necessary biosynthetic processes, such as lipid biosynthesis (61).

Lipid metabolism

Lipid peroxidation is considered the primary factor in ferroptosis. Lipid peroxides have the ability to destroy the stability of the lipid bilayer and thus the disintegration of cell membranes (62). Researchers have suggested that polyunsaturated fatty acids (PUFAs) are susceptible to lipid peroxidation, possibly due to the presence of highly active hydrogen atoms in methylene bridges. Hydroxyl radicals exert direct effects on the formation of lipid peroxides by interacting with PUFAs in membrane phospholipids, which then attack the cytomembrane and trigger ferroptosis (63). Furthermore, nucleic acids and proteins react with derivatives produced by the decomposition of lipid peroxides, such as 4-hydroxynonenal (4-HNE) and malondialdehyde (MDA), which could also lead to cell destruction (64). These derivatives, which act as markers, could also be useful for the detection of ferroptosis and lipid peroxidation. NADPH (nicotinamide adenine dinucleotide phosphate)–cytochrome P450 reductase transfers electrons from NAD(P)H to oxygen to generate hydrogen peroxide, which subsequently reacts with iron to generate reactive hydroxyl radicals for the peroxidation of the PUFA chains of membrane phospholipids, thereby disrupting membrane integrity during ferroptosis (65). In addition, ferroptosis is promoted by LOX-catalyzed lipid hydroperoxide generation in cellular membranes (66). Mechanistically, AMPK regulates ferroptosis through acetyl-CoA carboxylase (ACC) and PUFA biosynthesis (67). Exogenous monounsaturated fatty acids (MUFAs) potently inhibit ferroptosis. This effect requires MUFA activation by acyl-coenzyme A synthetase long-chain family member 3 (ACSL3) and is independent of lipid droplet formation (68). In addition, lipid peroxidation is inhibited by Fer-1, liproxstatin-1 (Lip-1), and vitamin E, which are free radical scavengers that reduce lipid peroxidation and effectively block ferroptosis (69, 70). Ferroptosis suppressor protein 1 (FSP1) suppresses ferroptosis independent of GSH. In the presence of NADPH, FSP1 reduces ubiquinone, also called coenzyme Q10 (CoQ10), to ubiquinol, which can reduce lipid peroxidation and promote ferroptosis (71). The synthesis and recycling of tetrahydrobiopterin (BH4) is a dynamic process, and GTP cyclohydrolase-1 (GCH1) is the rate-limiting enzyme in the biosynthesis of BH4. GCH1-mediated BH4 production prevents ferroptosis by inhibiting lipid peroxidation, which indicates that BH4 exhibits antioxidant activity during cell death (72).

Ferroptosis and MSKs

Osteoporosis

Osteoporosis is a common disease and a major public health concern, which has heightened the fracture risk with an increasing prevalence in elderly people regardless of sex or age. The homeostasis and integrity of bone tissue require a balance between osteoclast and osteoblast activity. In addition, the remodeling of bone tissue is a continuous and cyclic process. In recent years, much attention has been focused on ferroptosis in the pathogenesis of osteoporosis (Figure 4).

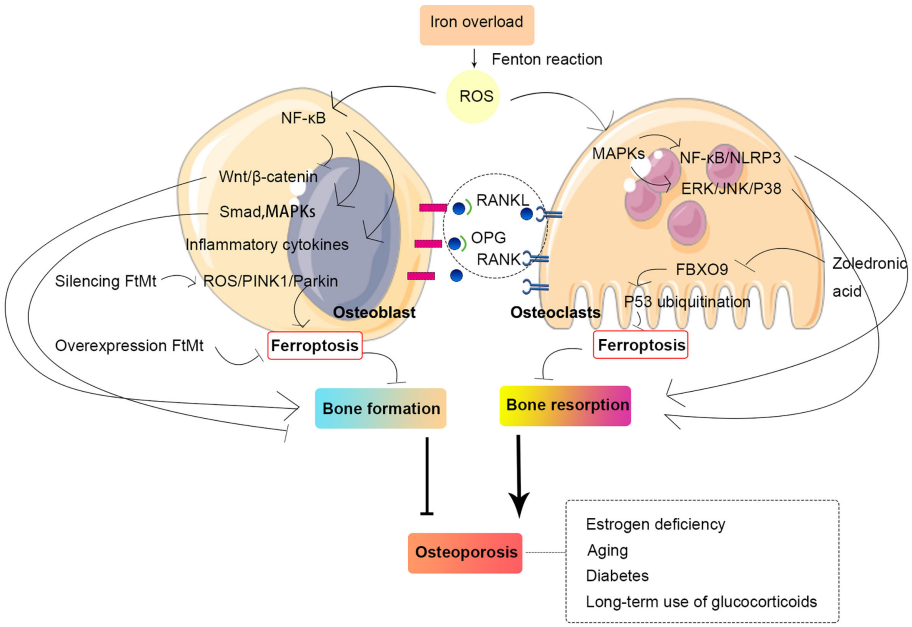

FIGURE 4
Relationship between osteoporosis and ferroptosis in osteoblast and osteoclast. The homeostasis and integrity of bone tissue require a balance between osteoclast and osteoblast activity. Iron overload can generate ROS through the Fenton reaction, and ROS can activate multiple intracellular signaling pathways, which, in turn, promote bone resorption and inhibit bone formation, thus leading to osteoporosis.
Abbreviations: MAPK, mitogen-activated protein kinase; NF-κB, nuclear factor-kappa B; FtMt, mitochondrial ferritin; PINK1, PTEN-induced kinase 1; ROS, reactive oxygen species; OPG, osteoprotegerin; RANKL, receptor activator for nuclear factor-κB ligand; RANK, receptor activator for nuclear Factor-κB; NLRP3, NOD-like receptor family pyrin domain containing 3; ERK, extracellular signal–regulated kinase; JNK, c-Jun N-terminal kinase; FBXO9, F-box only protein 9.

Ferroptosis occurs in osteoclasts

In terms of physiological characteristics, osteoclasts are multinucleated giant cells formed by the fusion of monocyte/macrophage precursor cells derived from myeloid progenitor cells in bone marrow with the indispensable involvement of macrophage colony-stimulating factor (M-CSF) and RANKL.

RANKL is stimulated by increasing the expression of the prostaglandin endoperoxide synthase 2 gene and MDA in bone marrow–derived macrophages (BMDMs) and decreasing GSH and iron levels, and iron accumulation is observed in mitochondria. ROS activate intracellular MAPK signaling pathways. ROS/MAPKs/nuclear transcription factor-kappa B (NF-κB)/NLRP3 activation causes osteoclast-mediated bone loss in diabetic osteoporosis (73). The activation of extracellular signal–regulated kinase, c-Jun N-terminal kinase (JNK), and P38 in the MAPK pathway can promote osteoclastogenesis, which leads to increased bone resorption (74). A recent study confirmed that zoledronic acid exerts ferroptosis-induced effects on osteoclasts by triggering FBXO9-mediated p53 ubiquitination and degradation (75). The inhibitory effect of artemisinin (ARS) compounds on osteoclast differentiation appears to be due to its downregulation of pathways involved in RANKL-induced osteoclastogenesis. In addition, mechanisms associated with intracellular iron, such as the cleavage of endoperoxide bridges, oxidative damage, and ferroptosis, are involved in the inhibition of osteoclast differentiation (76).

Ferroptosis occurs in osteoblasts

Osteoblasts play an essential role in bone regeneration and play a leading role in the synthesis, secretion, and mineralization of the bone matrix (77). The inhibitory effect of iron on the osteogenic differentiation of MSCs has been described, and iron overload in mice is correlated with increased ferritin and decreased RUNX family transcription factor 2 (RUNX2) levels in compact bone osteoprogenitor cells (78).

NF-κB induces inflammatory factors, inhibits Wnt signaling, and activates Smad and MAPK signaling pathways in osteoblasts to inhibit osteogenic differentiation (79, 80). Mitochondrial ferritin (FtMt) is a protein that stores iron and intercepts toxic ferrous ions in cellular mitochondria. Many studies have shown that FtMt reduces oxidative stress and maintains intracellular iron homeostasis (81). The overexpression of FtMt reduces ferroptosis in osteoblasts under high-glucose conditions,

whereas the silencing of FtMt can induce mitochondrial autophagy through the ROS/PINK1/Parkin pathway, which leads to increased ferroptosis in osteoblasts (82).

A previous study revealed that iron overload inhibits MC3T3 cell viability and induces apoptosis (83). This result suggests that iron overload may inhibit the activity of osteoblasts to some extent and thereby affects their differentiation and mineralization processes. Iron overload leads to excessive ROS and thus activates intracellular signaling pathways that affect cellular activity (84). A high dose of dexamethasone (10 μM) exerts its ferroptosis-induced effects on osteoblasts and thus downregulates GPX4 and system xc- (85). After induction with high glucose, MC3T3 cells, which exhibit increased ROS and reduced GPX4, have mitochondria that are generally smaller and less tubular, and the membrane exhibited darker staining and distinctly disrupted membrane folding. In a high-glucose environment, MC3T3 cells could the ability to reduce differentiation into osteoblasts and form mineralized nodules, as has similarly been observed in osteoblasts in mice (82, 86). In addition, it has been widely reported that advanced glycation end products play an important role in OP, particularly in diabetes–related OP, which may be caused by disruption of osteoblast functions by the induction of ferroptosis (87).

Osteoarthritis

OA is a common disease that leads to pain, acute care hospitalizations, disability, and socioeconomic costs worldwide (88). Because of better understanding of its pathogenesis, the focus of treatment is shifting to the prevention and treatment at early stages. Studies have shown that OA does have some features in common with ferroptosis, such as lipid peroxidation (89), glutathione oxidation (90), mitochondrial dysfunction (91), and increased activities of lipoxygenase and cyclooxygenase in chondrocytes of OA cartilage (92).

Late-response upregulated genes are strongly enriched in the ferroptosis pathway in an *in vitro* model of OA treated with fibronectin fragments (FN-f) (93). A study pointed to a progressive increase in the sensitivity of chondrocytes to oxidative stress with decreases in GPX4, which suggests the role of GPX4 in ferroptosis in OA. In addition, GPX4 can aggravate extracellular matrix degradation through the MAPK/NF-κB pathway (94). Furthermore, Guo Z et al. (95) found that increased MMP13 expression and decreased collagen II expression in chondrocytes can be stimulated by chondrocyte ferroptosis.

Various novel treatments that inhibit ferroptosis are being extensively researched. For example, DFO has the ability to inhibit chondrocyte ferroptosis and promote NRF2 antioxidant system activation (96), which are essential for the protection of chondrocytes. In addition, activation of the system xc-/Gpx4 axis by icariin treatment can inhibit Gpx4, SLC7A11, and SLC3A2L expression and reduce TFR1 expression, which significantly reduces the induction of cell death and inhibits ferroptosis (97). Zhou et al. (98) found that D-mannose could alleviate OA progression *via* HIF-2α–mediated inhibition of the sensitivity of chondrocytes to ferroptosis. In addition, stigmasterol reduces IL-1β–induced damage and ferroptosis in ATDC5 cells through sterol regulatory element–binding transcription factor 2, which can also enhance the inhibitory effect of ferroptosis inhibitors on injury (99). The relationship between ferroptosis and OA is shown in Figure 5.

Rheumatoid arthritis

RA is a relatively systemic common autoimmune disease with manifestations that include irreversible peripheral joint destruction and functional loss (100). Both genetic and environmental risk factors participate in the development of RA (101).

Oxidative stress and subsequent ROS-mediated cell death have recently been found to possibly play a critical role in RA development. Some evidence suggests that patients with RA with high persistent activity have reduced GPX activity in polymorphonuclear leucocytes (102). In addition, RSL3 can aggravate synovitis by inducing ferroptosis in synovial cells (97). Glycine can increase the S-adenosylmethionine (SAM) concentration to modulate the ferroptosis pathway by promoting SAM-mediated methylation of the GPX4 promoter and reducing FTH1 expression in RA fibroblast-like synoviocytes (103).

It has been indicated that serotransferrin-related molecules may be a promising method for investigating refractory RA (104). In addition, Tumor necrosis factor (TNF) signaling exerts its effect on fibroblasts in different manners to protect them from ferroptosis, such as promoting cystine uptake and increasing the biosynthesis of GSH (105). Moreover, studies have shown that CoQ10, a fat-soluble antioxidant, is a crucial regulator of ferroptosis. Jhun et al. (106) used CoQ10-encoded liposome/gold hybrid nanoparticles to attenuate RA progression by targeting signal transducer and activator of transcription-3/T helper cell 17 (STAT3/Th17).

Intervertebral disc degeneration

Intervertebral disc degeneration (IVDD) is a complex pathological condition caused by intractable back pain or secondary neurological deficits involving age-related changes and tissue damage produced by multiple stresses (107, 108). Because there is currently no fundamental treatment for the disease, an indirect symptom relief strategy has been employed. Of note, increasing evidence suggests that ferroptosis is involved in IVDD, which provides a new direction for therapeutic targets.

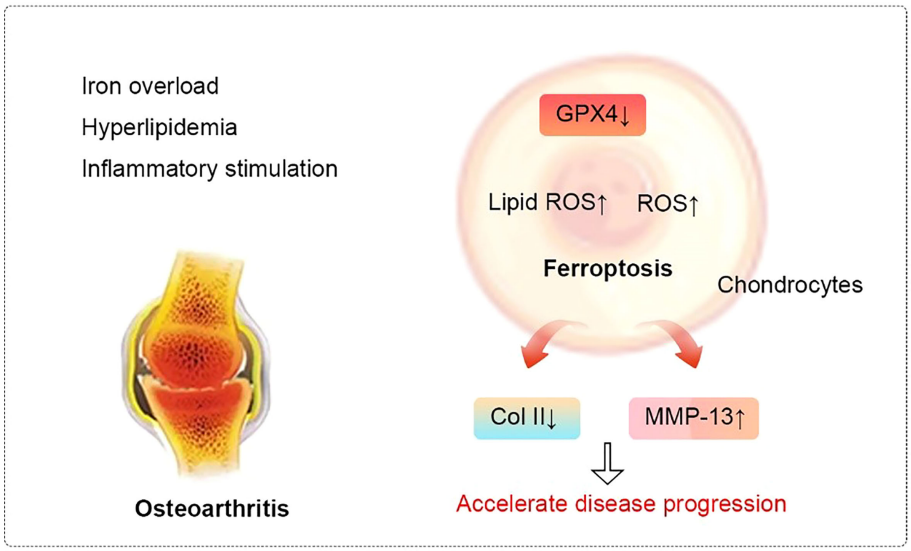

FIGURE 5
Relationship between ferroptosis and osteoarthritis. In cellular environments stimulated by iron overload, hyperlipidemia, and inflammation, the expression of Gpx4 in chondrocytes decreases. These changes lead to the accumulation of reactive oxygen species and lipid peroxides to ultimately induce ferroptosis. Ferroptosis, in turn, can progressively exacerbate the inflammatory response, leading to the increased expression of MMP-13 and decreased expression of collagen II in chondrocytes to accelerate the progression of OA. Abbreviations: ROS, reactive oxygen species; Col II, Type II collagen; MMP-13, matrix metalloproteinase-13.

Zhang et al. (109) previously demonstrated that ferroptosis is involved in IVDD. Ferroptosis in related tissue may appear to be due to exposure to high levels of heme, which may be caused by neovascularization in the prominent nucleus pulposus, and the progressive degeneration of herniated nucleus pulposus is also accelerated (110). Moreover, the levels of GPX4 and FTH1 in the degenerated disc tissues of IVDD rats are lower than those in those of healthy rats (111). Studies have also shown that the pathogenesis of IVDD involves ferritin degradation mediated by ferritin phagocytosis and subsequent lipid peroxidation. The disruption of iron homeostasis in degenerative disc tissue may be driven by increased levels of autophagy and NCOA4-regulated ferritinophagy upon exposure to tert-butyl hydroperoxide. In addition, homocysteine, as a novel contributor to IVDD, exerts its effects on oxidative stress and ferroptosis in the nucleus pulposus by enhancing GPX4 methylation (112).

An increase in the nuclear translocation of metal-regulated transcription factor 1 is achieved by restoring FPN function, eliminating intercellular iron overload, and thus protecting cells from ferroptosis. In addition, the process can be enhanced by hinokitiol through inhibition of the JNK pathway, which results in improving the progression of IVDD *in vivo* (113). It has been confirmed that IL-6 and its receptor led to chondrocyte ferroptosis by inducing cellular oxidative stress and interfering with iron homeostasis, which can be inhibited by overexpression of miR-10a-5p, and these findings suggest that the IL-6/miR-10a-5p/IL-6R axis could be a potential therapeutic target for intervention in IVDD (114).

Sarcopenia

Sarcopenia is defined as low muscle mass together with low muscle function. Diseases that can lead to secondary sarcopenia include malignancies, chronic obstructive pulmonary disease, heart failure, and kidney failure. Thus, further research is needed for the development of appropriate methods for the management of sarcopenia (115).

Soaring evidence supports the role of ROS accumulation and decreased endogenous antioxidant mechanisms in the progression of sarcopenia (116–118). Previous studies have reported associations among muscle iron accumulation, ROS production, and muscle wasting (119–121). Studies have also revealed that skeletal muscle atrophy induced by iron overload is related to ROS-mediated ubiquitin–proteasome system activation (122). Notably, iron plays a crucial role in ferroptosis triggered by P53-Slc7a11 in muscles, which suggests a therapeutic strategy for targeting iron accumulation (123). Interestingly, the release of iron mediated by macrophages can facilitate muscle regeneration (124), whereas oxidative stress and skeletal muscle atrophy have been found in mice with chronic iron injection for 14 days (125). In addition,

supplementation with DFO, an iron chelating agent, has the ability to reduce oxidative stress and inflammation in the diaphragm muscle of mice with Duchenne dystrophy (126). The latest research has shown that satellite cell–specific deletion of TFR1 impairs skeletal muscle regeneration by activating ferroptosis (127). Given the effect of iron on ROS production, the role of iron in the homeostasis of muscle satellite cells deserves more attention.

Rhabdomyolysis

Rhabdomyolysis (RM) is a common disease associated with myoglobinuria, electrolyte abnormalities, and acute kidney injury (AKI). The aims of the related treatments are to stop further skeletal muscle damage, prevent acute renal failure, and rapidly detect potentially life-threatening complications (128, 129).

A recent study has implicated ferroptosis in RM-related kidney damage *in vivo* and *in vitro* (130). RM results in the release of muscle cell components, including myoglobin, into the bloodstream, and the resulting myoglobin is freely filtered by the glomerulus and reabsorbed by the proximal tubule, which results in the promotion of ferroptosis-mediated cell death and leads to AKI. Moreover, the severity of AKI can be inhibited by Fer-1 treatment through the reduction of myoglobin-derived iron accumulation and lipid peroxidation (131). In animal models, iron chelators also have the ability to protect functional and histologic RM (132). Another previous study indicated the protective role of ACSL4 in mediating ferroptosis in the development of RM following EHS, which suggests that ACSL4 may also be a novel therapeutic target in RM (133). Overall, we point out that ferroptosis may play a vital role as a fundamental mechanism in a variety of MSKs (Figure 6).

Discussion

With advancing aging, the burden of MSKs will undoubtedly increase. In addition, the prevalence of people not paying attention to their diet, lifestyle, health, and physical activity contributes to the disease burden (134). Unclear pathogenic mechanisms for age-related MSKs challenge clinical practitioners (135). Raising the public awareness of risk factors and increasing their understanding by the medical and scientific community are pragmatic approaches to address these issues.

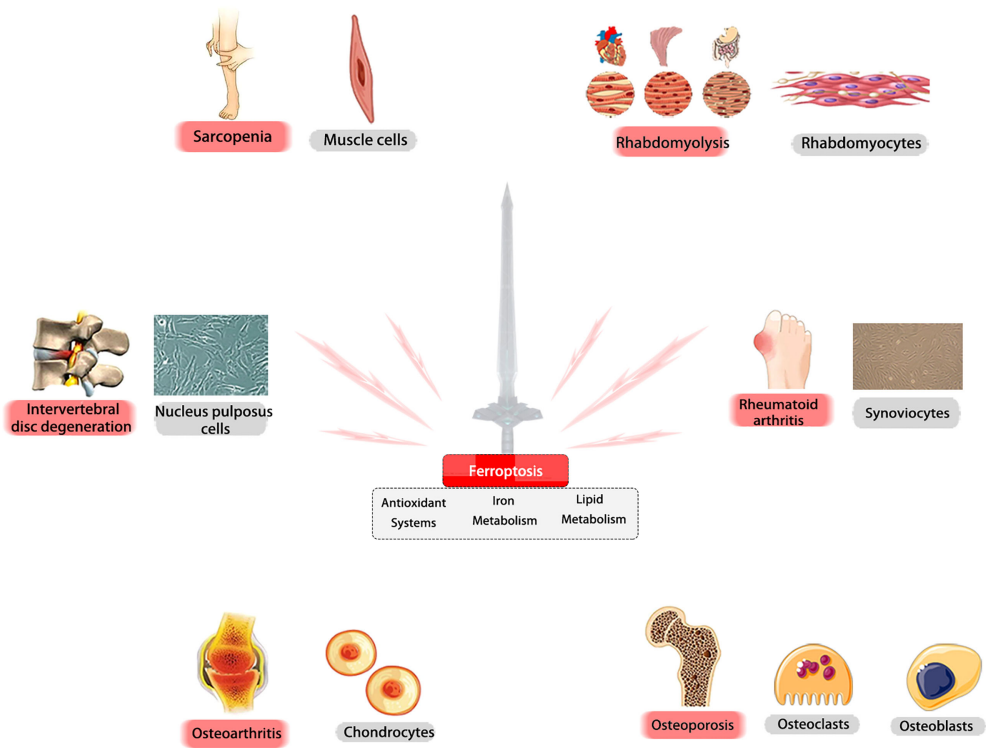

FIGURE 6
Ferroptosis may play a vital role as a fundamental mechanism in a variety of MSKs. Ferroptosis is critically involved in the pathogenesis of various MSKs. In different tissues and cells, ferroptosis -related molecules could be promising targets for treating these diseases.

Ferroptosis, a novel and unique form of RCD, is broadly involved in the development of cancers, kidney diseases, neurological diseases, and MSKs. In this review, we summarized the classic pathways of ferroptosis, including iron metabolism, antioxidant systems, and lipid metabolism, with a focus on affected disorders such as osteoporosis, OA, RA, IVDD, sarcopenia, and RM. Of note, iron, lipids, and ROS play an irreplaceable role in cell survival. However, excessive dependence is a double-edged sword. These three factors maintain normal body function in a steady state and strike a deadly blow to cells when metabolic disorders occur. The complex biological processes involved in ferroptosis are induced by an imbalance among antioxidants, iron, and lipid dynamics, but their role and contribution to the occurrence and metastasis of MSKs remain unclear.

Exploring the underlying mechanisms of ferroptosis in MSKs appears to have diverse favorable effects (136); however, before we move to related clinical applications, various issues need to be addressed. First, the fact that the current studies on ferroptosis and MSKs only scratch the surface of phenomena and results remains a challenge for precision medicine, and the detailed role of ferroptosis in the occurrence and development of MSKs has not been thoroughly studied. Second, most studies of MSKs have investigated ferroptosis only in cells and animal models and lack validated clinical evidence. Thus, clinical studies are imperative to solidify a conclusive role of ferroptosis in humans as soon as possible. Finally, ferroptosis appears to be a double-edged sword for MSKs. The inhibition of ferroptosis relieves the symptoms of osteoporosis or OA. However, the toxicity of ferroptosis-inhibiting or ferroptosis-inducing compounds on other organs is largely unclear. This evidence indicates that the role of ferroptosis is different in MSKs. Hence, although several characteristics and biomarkers of ferroptosis have been proposed, the specific and accurate quantification of the ferroptosis response, particularly *in vivo*, remains a major challenge.

In summary, ferroptosis is critically involved in the pathogenesis of various MSKs. With ongoing research, we point out that ferroptosis will be an area worthy of in-depth study and will play a vital role in the subsequent development and clinical translation of new drugs for MSKs.

Author contributions

XW and LZ were responsible for the conceptualization of the study. XH was responsible for the methodology. XH and YZ were responsible for the validation. XH and YZ completed the formal analysis. CS, BQ, and NL were responsible for the resources. KS completed the data curation. YZ and XH prepared the original draft. XW and LZ reviewed and edited the manuscript. All authors contributed to the article and approved the submitted version.

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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.

Publisher's note

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Glossary

MSKs	Musculoskeletal disorders
ACD	Accidental cell death
RCD	Regulated cell death
ROS	reactive oxygen species
OA	Osteoarthritis
RA	Rheumatoid arthritis
HO·	Hydroxyl radicals
TFR1	Transferrin receptor 1
STEAP3	Six-transmembrane epithelial antigen of prostate 3
DMT1	Divalent metal transporter 1
LIP	Labile iron pool
NCOA4	Nuclear receptor coactivator 4
FPN1	Ferroportin 1
FTH1	Ferritin heavy chain 1
FTL1	Ferritin light chain 1
Fer-1	Ferrostatin-1
DFO	Defetoxamine
GPX4	Glutathione peroxidase 4
GSH	Glutathione
GSSG	Oxidized glutathione
L-OOH	lipid peroxides
LOH	lipid hydroxyl derivatives
RSL3	RAS-selective-lethal-3
ATF4	Activation of transcription factor 4
system xc-	the cystine/glutamate antiporter system
NRF2	NF-E2-related factor 2
BAP1	BRCA-1-associated protein
ATF3	The activation of transcription factor 3
BECN1	beclin 1
TCA	Tricarboxylic acid
PUFAs	Polyunsaturated fatty acids
4-HNE	4-hydroxynonenal
MDA	Malondialdehyde
MUFAs	Monounsaturated fatty acids
ACSL3	acyl-coenzyme A synthetase long-chain family member 3
Lip-1	liproxstatin-1
FSP1	Ferroptosis-suppressor-protein 1
NADPH	Nicotinamide adenine dinucleotide phosphate
BH4	tetrahydrobiopterin
GCH1	GTP cyclohydrolase-1
M-CSF	Macrophage colony-stimulating factor
PTGS2	Prostaglandin endoperoxide synthase 2
BMDMs	Bone marrow-derived macrophages
DOP	Diabetic osteoporosis
ARS	Artemisinin
RUNX2	RUNX family transcription factor 2
FtMt	Mitochondrial ferritin
AGEs	Advanced glycation end products
FN-f	Fibronectin fragments
ECM	Extracellular matrix
STM	Stigmasterol
SREBF2	Sterol regulatory element binding transcription factor 2
SAM	S-adenosylmethionine
FLSs	Fibroblast-like synoviocytes
STAT3/Th17	Signal transducer and activator of transcription-3/T helper cell 17
IVDD	Intervertebral disc degeneration
MTF1	Metal-regulated transcription factor 1
AKI	Acute kidney injury

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The role of macrophage scavenger receptor 1 (MSR1) in inflammatory disorders and cancer

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Macrophage scavenger receptor 1 (MSR1), also named CD204, holds key inflammatory roles in multiple pathophysiologic processes. Present primarily on the surface of various types of macrophage, this receptor variably affects processes such as atherosclerosis, innate and adaptive immunity, lung and liver disease, and more recently, cancer. As highlighted throughout this review, the role of MSR1 is often dichotomous, being either host protective or detrimental to the pathogenesis of disease. We will discuss the role of MSR1 in health and disease with a focus on the molecular mechanisms influencing *MSR1* expression, how altered expression affects disease process and macrophage function, the limited cell signalling pathways discovered thus far, the emerging role of MSR1 in tumour associated macrophages as well as the therapeutic potential of targeting MSR1.

KEYWORDS

MSR1, CD204, macrophages, immunology, cancer, inflammation

Introduction

The innate immune response and the inflammatory response constitute the first mechanisms of host defence. Here, macrophages are crucial innate immune cells that play an essential role by maintaining tissue homeostasis and eliminating pathogens by phagocytosis, the uptake of particulate material (1) Macrophages are found in various tissues and display phenotypic heterogeneity depending on their tissue environment and activation state. The typical nomenclature used to describe macrophage activation splits macrophage populations into two broad states, M1 (classically activated) or M2 (alternatively activated). These states are defined by inflammatory status. M1 macrophages are pro-inflammatory, specialising in pathogen killing whilst M2 macrophages are anti-inflammatory and are responsible for tissue repair and the promotion of cell proliferation (2, 3). This approach to defining macrophages is, however, reductionist and often leads to confusion. It is therefore important to

recognise that macrophages exist on a spectrum of activation states, rather than in a binary system (3). Macrophage scavenger receptor 1 (MSR1) has been shown to be important for M2 polarisation (4). Therefore, the role it carries or MSR1 is in part determined by the type of macrophage in which MSR1 is expressed and location within which the macrophages reside.

MSR1, also known as scavenger receptor-A (SR-A) or cluster of differentiation 204 (CD204), was first described in 1979 by Brown and Goldstein (5). They demonstrated that MSR1 mediated the uptake and degradation of acetylated low-density lipoprotein (acetyl-LDL) but not non-modified low-density lipoprotein (LDL). This led to increased intracellular cholesterol deposition, and they postulated that the receptor is responsible for similar effects observed in familial hypercholesterolemia. Further research identified pathologic similarities between lipid-laden cells and foam cells found in the tissue of hyperlipidaemic patients (6). Therefore, the first role described for MSR1 was in the pathogenesis of atherosclerosis, which kick-started further investigations into MSR1. Whilst MSR1 has been shown to be active across the disease spectrum, our understanding of the molecular mechanisms behind its various functions has been marred by incomplete signalling pathways and contradictory experimental results. This therefore introduces a complex view of the role MSR1 plays in macrophage-mediated inflammatory disorders.

In this review we will outline the genetic, epigenetic, and post-translational alterations affecting MSR1, as well as the molecular mechanisms and cell signalling of MSR1. Finally, our interest is to collate the evidence available regarding the clinical implications of MSR1 alterations in different pathologies of the immune system and its role in tumour-associated macrophages (TAMs) in cancer, especially as it has been proposed as a potential biomarker with prognostic value. Finally, we will provide an overview of future avenues for therapeutic targeting of MSR1.

MSR1 structure and ligand recognition

As more scavenger receptors with similar broad binding specificities were discovered, they were first subdivided into "classes", based on their primary sequences, and then further subdivided into "types" as a result of sequence variation caused by alternative splicing. This subdivision gave rise to the scavenger receptor family which currently consists of twelve different classes (classes A-L), grouped together by shared functional and ligand binding properties rather than sequence homology (7–9) (Figure 1).

The class A scavenger receptors, including MSR1, share a similar ligand repertoire due to the presence of a collagen-like binding domain. These receptors are expressed in a range of different organs but work mainly to clear bacteria, bind and degrade modified lipids, and in the case of SR-A3, protect against reactive oxygen species. Class B scavengers bind high density lipoprotein and influence the development of atherosclerosis. SR-B1 has a protective effect whilst CD36, which also binds oxLDL, has been shown to potentiate atherosclerosis. Expression of class C scavenger receptors is restricted to *Drosophila melanogaster*. CD68, a member of class D, contains a lysosome-associated membrane glycoprotein (LAMP) domain and functions mainly to scavenge oxLDL. The class E scavenger receptors, characterised by presence of a C-type lectin-like domain, function primarily to recognise and remove pathogen associated molecular patterns (PAMPs). Class F scavenger receptors, including SREC-1, bind fungal pathogens, heat shock proteins (HSPs), and apoptotic cells. SR-PSOX belongs to class G and binds oxLDL, phosphatidylserine and mediates phagocytosis of bacteria. It is also the only protein known to share both scavenger receptor and chemokine activities. The class H receptors, FEEL-1 and FEEL-2, are phagocytic receptors that clear apoptotic cells and also mediate angiogenesis. Class I receptor CD163 is similar to other scavenger receptors in its ability to bind gram-positive and negative bacteria but is unique in its haematological role aiding clearance of plasma haemoglobin. RAGE, a member of class J, binds multiple ligands and is classed as a pattern recognition receptor during chronic inflammation or infection. RAGE signalling mediates oxidative stress, inflammation and apoptosis. The class K scavenger receptor CD44 is an endocytic receptor that binds hyaluronan and other components of the extracellular matrix. Finally, LRP1 is a low density lipoprotein receptor that functions primarily to clear plasma cholesterol (10). As is evident, scavenger receptor classes are highly similar in their ligand binding capabilities and function, with multiple classes able to bind and clear pathogens, PAMPS and modified self-molecules. Therefore the scavenger receptor family plays a significant role in host defence.

The *MSR1* gene consists of 11 exons and encodes the class A macrophage scavenger receptors (SR-A); three different isoforms are generated by alternative splicing: SR-AI, SR-AII and SR-AIII. SR-AI and SR-AII are the two predominant isoforms in mammals, the only difference between the two being the lack of a cysteine-rich C-terminus region in SR-AII (11, 12). SR-AIII is a truncated, non-functional variant that has altered intracellular processing and becomes trapped within the endoplasmic reticulum. Presence of this isoform can, however, reduce modified LDL uptake by SR-AI and SR-AII, suggesting a function as a dominant negative isoform as well as a mechanism for regulation of scavenger receptor activity in macrophages (13).

MSR1 is a homo-trimeric transmembrane glycoprotein consisting of six distinct domains, with the collagen-like domain and the scavenger receptor cysteine-rich (SRCR) domain being most relevant to its function (14) (Figure 2). The site for ligand interaction is found in the extracellular collagen-like domain. Lysine clusters within this domain form positively charged grooves allowing interaction with polyanionic ligands (15). The existence of binding sites within the collagen-like domain explains why SR-AI and SR-AII have identical binding capabilities despite the C-terminal differences.

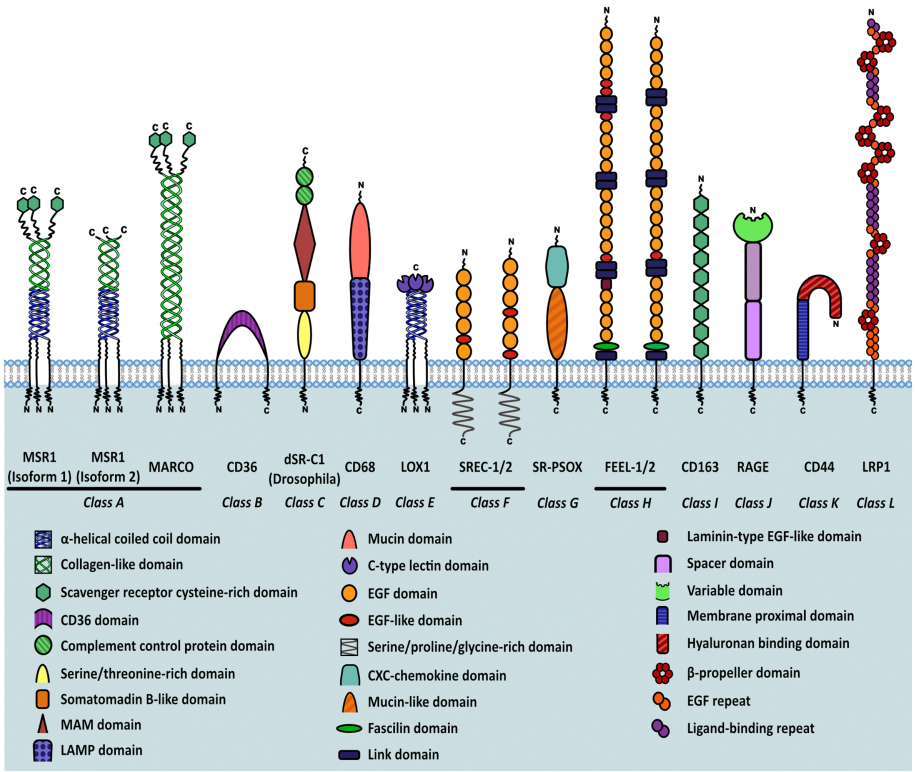

FIGURE 1
Classification of scavenger receptors and MSR1 isoforms. Schematic representation of different classes of scavenger receptors. Individual domains are identified in the key within the figure.

The ligand repertoire of MSR1 is an unusually diverse array of both endogenous and exogenous ligands (Table 1), thus leading to its description as 'molecular flypaper' (45). Interestingly, whilst all known ligands share the common feature of being polyanionic, not all polyanions are ligands. This suggests that whilst the possession of multiple negative charges is an absolute requirement, ligand structure also contributes to binding ability (46). Therefore, MSR1 behaves in some way as a pattern recognition receptor.

An interesting binding phenomenon also occurs within the collagen-like domain, referred to as nonreciprocal cross-competition of ligands. In simpler terms, if two different ligands are present, ligand A may be able to completely out-compete ligand B, but ligand B is unable to completely displace ligand A, even at significantly higher concentrations. When comparing the binding dynamics of oxidised-LDL and acetyl-LDL, this property becomes apparent. This has been attributed to the presence of two distinct but overlapping binding sites. Nonreciprocal cross-competition has interesting implications when considering the complex signalling surrounding MSR1 as the receptor may be unable to bind a high affinity ligand due to the pre-existing presence of a lower affinity ligand. Therefore, the order in which MSR1 binds its ligands may have an impact on signalling (47). However, this has yet to be experimentally demonstrated.

Interestingly, despite the receptor being associated with a diverse range of physiological and pathological processes, the cytoplasmic domain lacks a discernible signalling motif. Therefore, to successfully propagate an intracellular signal, MSR1 must associate with other membrane receptors, cytoplasmic components, or rely on post-translational modifications (PTMs) to enable the recruitment of signalling complexes (29, 48).

Genetic and epigenetic alterations affecting MSR1

MSR1 is highly expressed in lung, arteries, and adipose tissue (Figure 3A). This receptor can be also found at low levels in other cell types: vascular smooth muscle cells (51, 52), astrocytes (53, 54), murine embryonic fibroblasts (35), human lung epithelial cells (36), and a range of endothelial cells (48, 55–57). MSR1 is, as its name suggests, predominantly expressed in various macrophage cell types (Figure 3B) (5, 6) and is mainly altered in macrophage-associated physiological and pathological processes including

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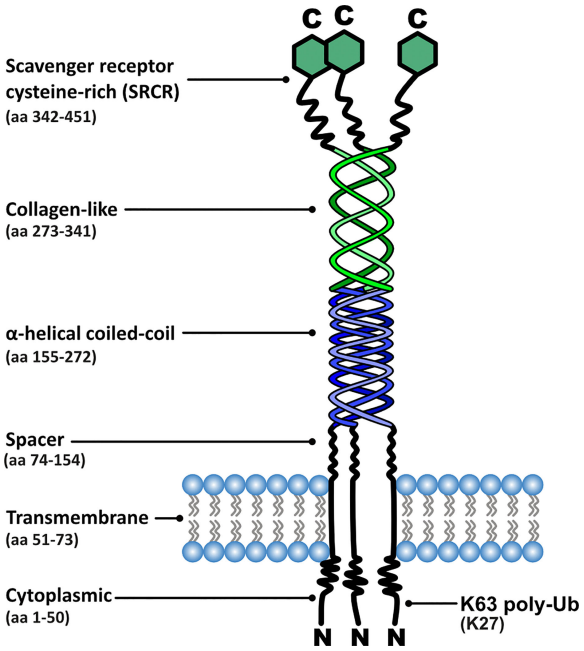

FIGURE 2
Functional domain organisation of MSR1, including corresponding amino acid (aa) positions and post-translational modification sites relevant to signalling.

atherosclerosis, Alzheimer's disease, host defence, and cancer. However, the mechanisms responsible for the alteration of MSR1 expression are not well-studied. Next, we will explore the different mechanisms responsible for changes in MSR1 expression.

Chromosome 8p deletion

A major event leading to under-expression of *MSR1* is DNA deletion (Figure 4). The *MSR1* gene is located on chromosome 8 in humans. The 8p arm is a 6.4 Mb-region that contains 20 other genes, besides *MSR1*, with high frequent loss of heterozygosity (LOH) and homozygous deletion (58). Deletion of this region has been frequently found in different types of human cancers and it is associated with disease progression and poor prognosis. Frequent gene deletions in this region are prognostic markers of cancer such as *HCRP1* (59) and *DLC1* (60) in hepatocellular carcinoma, or *TUSC3* in oral squamous cell carcinoma (61). Also, deletion of the microRNA miR-383 at 8p22 region leads to tumour initiation and prostate cancer metastasis (62). Deletions in this region are also linked with high aggressiveness and risk of disease progression of prostate cancer, even after a radical prostatectomy (63). Insertions into *MSR1* gene have also been observed in gastric adenocarcinoma (COSMIC ID: 8185658). As *MSR1* expression is already low in these cell types, it is unclear how *MSR1* deletion contributes to the progression of cancer. The changes in prognosis seen as a result of deletions in this region are therefore more likely due to loss of other genes in this region.

MSR1 mutations

Mutations in MSR1 are the second most frequent genetic alteration, which represent 3.3% (1250 out of 38170 tumour samples) according to the COSMIC (the Catalogue Of Somatic Mutations In Cancer) database. In the TCGA PanCancer Atlas Studies, a total of 220 different somatic mutations were identified, of which 35 affected the protein sequence and 3 involved MSR1 in fusion proteins (Figure 4). However, MSR1 was not catalogued as a cancer driver by the IntOGen-mutations platform (64). To date, 30,163 variants have been described in the Ensembl database. However, only 3 of them have been attributed to pathogenesis and clinical consequence, NM_138715.3 (MSR1): c.520G>T (p.Asp174Tyr) and c.877C>T (p.Arg293Ter) in prostate cancer (65); and c.760C>G (p.Leu254Val) specifically related to Barrett oesophagus and oesophageal adenocarcinoma (66).

Transcriptional regulation of MSR1

MSR1 transcriptional expression is regulated by different transcription factors, such as: SPI1, ETS2, c-JUN, c-FOX, and CEBPB (67–69); and predicted transcription factors (70), such as: STAT2, IRF5 and DNAJC2, which are shown in Figure 5. The activity of c-Jun and STAT2 in the regulation of MSR1 is interesting as the JAK/STAT and JNK/SAPK pathways are also involved in leptin-induced chemotaxis of monocytes, macrophages, and cancer cells (71, 72). This link may be important for the activity of MSR1 in the tumour microenvironment, as will be discussed later in this review. On the other hand, there are pro-inflammatory cytokines that inhibit MSR1 transcription or reduce MSR1 activity in macrophages, such as, the tumour necrosis factor alpha (TNF-α) (73), interferon-gamma (IFN-γ), and the transforming growth factor-1 (TGF-1) (74, 75). The transcription factors MITF, MAF, THRA, and NR1H3 were proven to bind around a single nucleotide polymorphism (SNP) (rs41505344) in the upstream transcriptional region of MSR1, suggesting an indirect role for the SNP in the transcriptional regulation of MSR1. Moreover, this SNP was associated with altered serum triglycerides and aspartate transaminase levels in non-alcoholic fatty liver disease (NAFLD) (76).

Regulation of MSR1 by microRNAs

Gene silencing by microRNAs (miRNAs) is another prominent mechanism of regulation, which combines translational repression

TABLE 1 List of the endogenous and exogenous ligands known to bind to MSR1 and their effects.

Endogenous ligand	Effects of ligand binding
Acetyl-LDL	Activation, inflammation, and pathogenesis of atherosclerosis (5, 16)
Advanced glycation and product modified proteins	Clearance and pathogenesis of atherosclerosis (17)
Apolipoproteins A-I (ApoAI) and E (ApoE)	Macrophage adhesion (18) Pathogenesis of atherosclerosis and Alzheimer's disease (18, 19)
Apoptotic cells	Clearance and reduction in inflammation (20)
β-amyloid	Microglia activation and inflammation (21) Protection against Alzheimer's (22)
Calciprotein particles	Clearance and protection against atherosclerosis (23)
Collagen	Macrophage adhesion (24)
Heat shock proteins (HSP)	Protection against atherosclerosis (25–27)
Maleylated LDL, HDL & albumin	Release of proteases (16)
Oxidised-LDL	Cholesterol deposition and pathogenesis of atherosclerosis (28) JNK-mediated inflammation (29)
Proteoglycans	Macrophage adhesion to extracellular matrix, and pathogenesis of atherosclerosis (30)
Exogenous ligand	**Effects of ligand binding**
Berberine alkaloids	Inhibition of LPS endocytosis (31)
Carrageenan	Used as a competitive inhibitor (16, 32)
Crocidolite asbestos	Possible link to asbestosis and mesothelioma (33)
Dextran sulphate	Used as a competitive inhibitor (16)
Double stranded RNA (dsRNA)	Sensing of, and protection against viruses (34–36)
Fucoidan	Used as a competitive inhibitor (16) JNK-mediated inflammation (29)
β-Glucans	Recognition of fungi and bacteria (37)
Human cytomegalovirus (HCMV)	Clearance and protection (38)
Lipopolysaccharide (LPS)	Clearance and protection against bacteria (39)
Lipoteichoic acid (LTA)	Clearance and protection against bacteria (40)
Muramyl dipeptide	NOD2-mediated inflammatory response (41)
Organic dust extract	Regulation of respiratory inflammation (42)
Polyguanylic (poly (G)) and Polyinosinic acid (poly(I))	Used as a competitive inhibitor (16)
Polyinosinic: polycytidylic acid (poly (I:C))	Used as a competitive inhibitor (16)
Silica	*In vitro* – silica induced apoptosis (43) *In vivo* – Appropriate regulation of immune response to silicosis (44)

and mRNA destabilization of target genes (77). Expression levels of MSR1 correlated with the expression of miRNAs that potentially target MSR1 during the differentiation and maturation of bone marrow derived macrophages (BMDMs) (78). Among these miRNAs we found: miR-24, miR-18b, miR-141, miR-150, miR-155, and let-7e. Inflammatory activation by Toll-like receptors (TLRs) leads to the expression of miR-155 in macrophages and dendritic cells (DCs) (79–81). These studies suggest that a wide range of inflammatory mediators can activate miR-155 through mechanisms mediated by both transcription factors: AP-1 and NF-κB. The predominant gene targeted by miR-155 in myeloid cells was the homeobox gene, HOXA9 (82), in DCs was CD274, encoding the inhibitory receptor ligand PD-L1, and in macrophages MSR1 (83). It has also been described that macrophage NFATc3 acts to upregulate miR-204 which in turn depletes levels of MSR1 and protects against atherosclerosis by limiting lipid uptake and subsequent foam cell formation (84).

Methylation of the MSR1 promoter region and histone modification

Methylation of gene promoters usually leads to transcriptional silencing. Although the effects of MSR1 promoter methylation are not well studied in pathologies associated with MSR1 deficiency, a negative correlation between MSR1 promoter DNA methylation and gene expression in monocytes has been described (85). In a genome-wide analysis of epigenetic and transcriptional variations, MSR1 was studied in terms of gene expression, methylation, and histone variation in human monocytes, neutrophils, and T-cells (86).

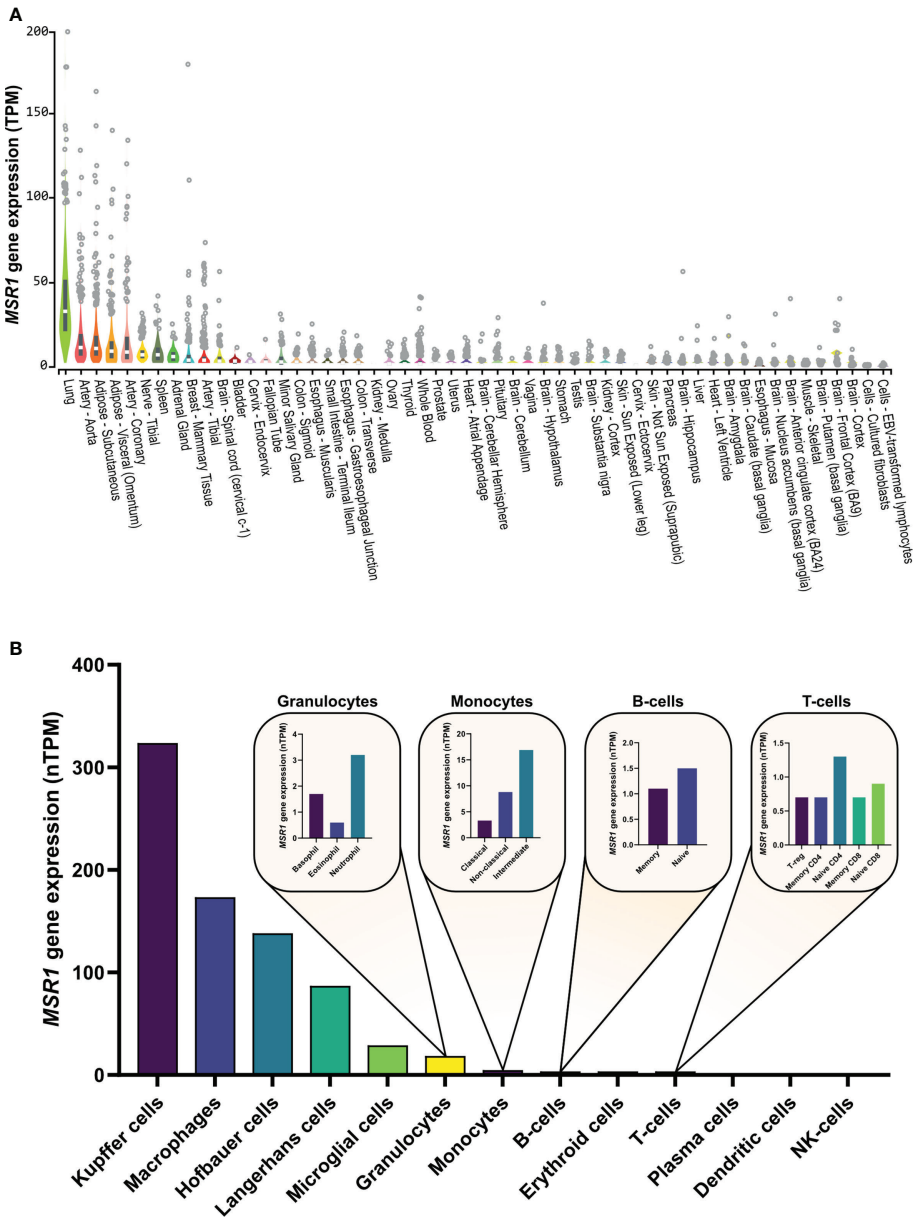

FIGURE 3
MSR1 expression in **(A)** normal human tissues and **(B)** human immune cells. Human tissue expression measured by RNA sequencing abundance of *MSR1* transcripts, using a normalization method to calculate transcripts per million (TPM), determined by the GTEx Project (49). Human immune cell *MSR1* expression measured by RNAseq as part of the human protein (HPA) database (proteinatlas.org) (50).

They described a robust contribution of cis-genetic factors (promoter methylation, H3K4me3, and H3K27ac) to the transcription of MSR1 in immune cells.

In summary, MSR1 expression is frequently altered in cancer, but specifically in cells of the immune system. Decreased expression is more frequently related to DNA deletion, although epigenetic alterations are also observed. However, high levels of MSR1 are found in tumour-associated macrophages (TAMs) as will be discussed at the end of this review

Cellular signalling pathways of MSR1

Ligand binding to MSR1 results in receptor internalisation, recruitment of specific binding partners, and the activation of downstream signalling pathways. Which pathway becomes stimulated is both ligand-dependent and macrophage activation state-dependent and whilst a detailed overall pathway surrounding MSR1 is elusive, some signalling cascades have been elucidated, albeit incomplete. Receptor

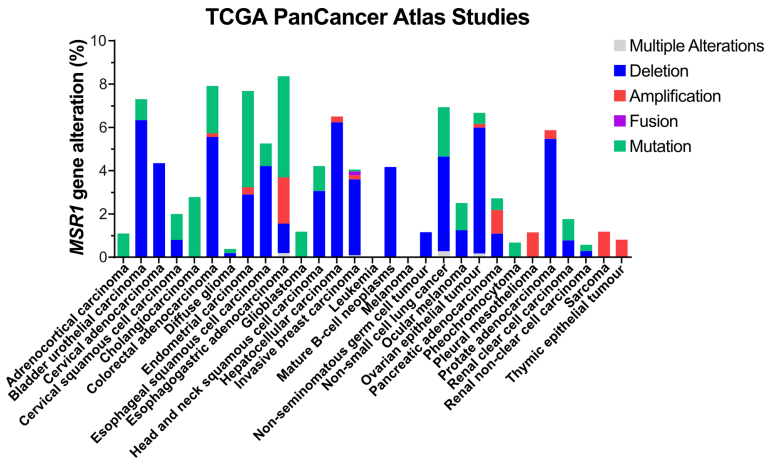

FIGURE 4
Genetic alterations of MSR1 in human tumour tissues. Data obtained from TCGA PanCancer Atlas Studies in 10967 tumour samples from various origins.

signalling has been most studied in macrophages, these include the fucoidan-induced signalling pathways PTK(Src)/Rac1/PAK/JNK and PTK(Src)/Rac1/PAK/p38 which act to regulate IL-1 secretion (87), and the MAP kinase pathway which induced TNF-α production (88) (Figure 6). It is worth noting that neither of these studies could explicitly state how MSR1 activates the resulting pathway. Instead, it was hypothesised that other receptors, heat shock proteins or tyrosine kinases located within the cytoplasm act as binding partners to propagate the signal.

The binding of fucoidan, but not other classical endogenous MSR1 ligands, also stimulated nitric oxide production. This signal was transduced by both p38 mitogen-activated protein kinase (MAPK) and NF-κB-dependent pathways directly downstream of HSP90, which interacted with the cytoplasmic domain of MSR1 (89). The exact cascade involved in either pathway was not interrogated here, therefore it is unclear which proteins are involved post-HSP90 recruitment to MSR1. However, it is possible that the PTK(Src)/Rac1/PAK/p38 pathway is involved (87). Another p38-MAPK mediated signalling response, also stimulated by fucoidan, was found to propagate *via* major vault protein (MVP) binding and the caveolin-mediated endocytic pathway (90). However, as MSR1 has no signalling domains on its short cytoplasmic domain, it remains unclear how binding partners interact. Furthermore, it is not clear how different signalling pathways have been elucidated after binding of the same ligand. Finally, MSR1 bound to human cytomegalovirus (CMV) in monocytes, interacting with toll like receptor (TLR) 3 and 9 on the endosome to stimulate both the IRF3 and NF-κB pro-inflammatory pathways (38).

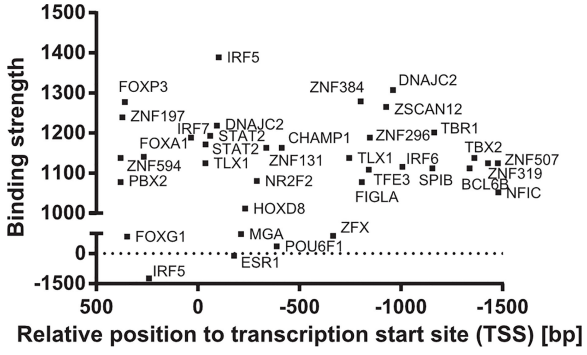

FIGURE 5
Transcription factors of *MSR1* promoter. Binding sites of transcription factors in the promoter of *MSR1* from experimental and theoretical sources from the TF2DNA database (70).

Our group conducted a study which focused on the dynamic protein changes using proteomics within the phagosomes of M2 macrophages. This unveiled one of the elusive molecular links between MSR1 and its downstream effectors. When triggered by negatively charged polystyrene beads, phagocytosis is induced and MSR1 localises to the phagosome, which acts as a signalling platform providing interaction with innate immune signalling networks. Upon triggering, MSR1 becomes polyubiquitylated at K27 with lysine-63 (K63) chains by an unidentified E3 ubiquitin ligase (Figure 7). This polyubiquitylation serves as a scaffold for the recruitment of the TAK1/MKK7/JNK signalling complex (29). Moreover, it has recently been described that MSR1-mediated uptake of saturated fatty acids can also activate JNK signalling and induce Tnf-α and Il-6 expression (76). Triggering of this

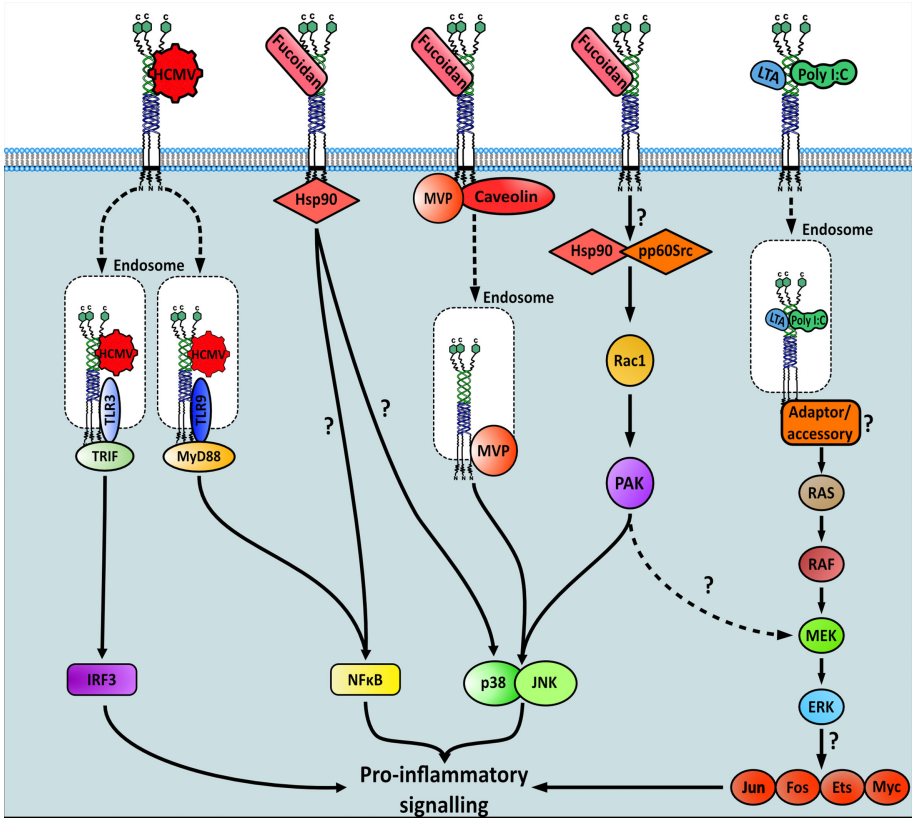

FIGURE 6
Signalling pathways downstream of MSR1 initiated after specific ligand binding. Question marks within the figure highlight gaps in the knowledge regarding each pathway.

pathway leads to a phenotype switch from anti-inflammatory to pro-inflammatory. Furthermore, MSR1 K63-polyubiquitin mediated JNK signalling was demonstrated in ovarian cancer patients, indicating a potential role for MSR1 (29). Due to the direct link between MSR1 and the downstream effectors this is one of the most complete signalling pathways surrounding MSR1 elucidated to date (Figure 7).

Post-translational modifications (PTMs) play important regulatory roles in biological processes, influencing protein functions, stability, and localisation. However, there is limited information regarding MSR1 regulation by PTMs other than polyubiquitylation as described above. N-glycosylation is one of the most prominent post-translational modifications and is involved in many cellular functions including receptor-ligand interactions, immune response, and pathogenesis of many diseases (91). Two glycoproteomics analyses identified N-glycosylation of MSR1 at N102, N221, and N249 in human diffuse large B-cell lymphoma (92) and in human liver tissue (93). MSR1 glycosylation appears to be relevant to its function since the N102K mutation has been linked to oral squamous cell carcinoma (COSMIC ID: 3715901).

Clinical implications of MSR1 alterations

MSR1 is highly pleiotropic, functioning in various physiological and pathological processes throughout diverse tissues (Figure 8). MSR1 acts as a double-edged sword in many processes by either protecting or damaging the body, determined by a plethora of variables, and working through mechanisms many of which remain poorly defined.

Atherosclerosis

Macrophages in atherosclerotic lesions actively participate in lipoprotein uptake and accumulation, giving rise to foam cells. The most extensively covered role of MSR1 is its activity in atherosclerosis, yet despite the abundance of investigations in this area there is ongoing debate regarding the true role of the receptor. It was first shown to be responsible for the uptake of modified lipoproteins and secretion of inflammatory cytokines leading to atherosclerotic lesion formation (5, 16). However,

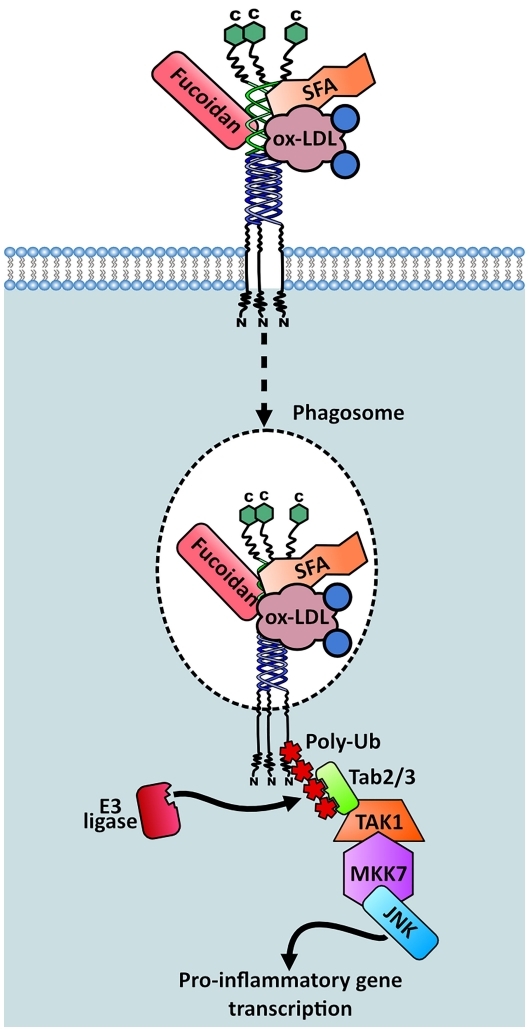

FIGURE 7
Signalling complex recruitment to MSR1 in M2 macrophages. Triggering of MSR1 by fucoidan, oxidised LDL (ox-LDL) or saturated fatty acids (SFAs) induces MSR1 K63 polyubiquitylation at K27, mediated by an unknown E3 ligase. This polyubiquitin chain acts as a scaffold for recruitment and activation of the TAK1/MKK7/JNK signalling complex. This stimulates a pro-inflammatory phenotypic switch within M2 macrophages.

MSR1 is not the sole receptor responsible for this. Macrophage uptake of modified LDL is mediated through scavenger receptors, mainly CD36 and MSR1, causing accumulation of large amounts of cholesterol within the macrophage. However, MSR1 was found to be more responsible for the binding and degradation of Acetyl-LDL, with CD36 more responsible for oxidised-LDL clearance (94). MSR1 led to greater modified LDL degradation than CD36, indicating that MSR1 may be more implicated in the pathologic breakdown and subsequent cholesterol deposition of modified LDL.

The function of MSR1 in the pathogenesis of atherosclerosis appears to be dependent on the mouse genetic background and apoE presence. However, due to conflicting findings, it is difficult to arrive at a unified hypothesis. *Msr1*$^{-/-}$ mice displayed a decrease in atherosclerotic lesion area compared to wild type (95). Similarly, *Msr1*$^{-/-}$ *ApoE-/-* mice also exhibited a decrease in lesion size (96). Also, in *Msr1*$^{-/-}$ *Ldlr*$^{-/-}$ mice the sizes of atherosclerotic lesions were reduced (19). However, this decrease was less dramatic and would indicate that *ApoE* may be a contributing factor rather than *Ldlr*. For this reason, other scavenger receptors, such as MARCO, CD36, and/or CD68, as well as uptake of very low-density lipoprotein (VLDL) might be involved in foam cell formation during atherogenesis in these mice. In stark contrast to these findings, *ApoE*$^{-/-}$ *Msr1*$^{-/-}$ mice fed an atherogenic diet still generated an abundance of foam cells associated with increased aortic lesion area (97). Similarly, *ApoE*$^{-/-}$ *Cd36*$^{-/-}$ *Msr1*$^{-/-}$ mice also showed no decrease in foam cell formation or lesion area. However, a slowed progression towards more advanced necrotic lesions was apparent, accompanied by a decrease in pro-inflammatory gene expression. This study also provides evidence against receptor compensation by CD36 being a cause of variation between investigations (98). Furthermore, mice with a dysfunctional variant of *ApoE*, ApoE3-Leiden, suffered increased susceptibility to atherosclerosis in a similar manner to complete loss of *ApoE*. Interestingly, ablation of *Msr1* also had no significant effect on lesion formation with trends towards more severe lesions apparent (99). Due to disparities in experimental results so far, the role that MSR1 plays in atherosclerosis is still controversial.

Nevertheless, several mechanisms pertaining to the activity and regulation of MSR1 in atherosclerosis have been elucidated in recent years. Perhaps a less obvious protective role played by MSR1 is the uptake of calciprotein particles and mineral debris from the blood circulation, this prevents soft tissue calcification and thus aids in the prevention of calcifying atherosclerosis (23). Intermedin (adrenomedullin-2), a cardiovascular protective peptide often found in atherosclerotic plaques, acts as a negative regulator of *MSR1* expression. Intermedin reduced the uptake and degradation of acetyl-LDL by macrophages, thus decreasing intracellular cholesterol levels. This was due to the increased phosphorylation and decreased ubiquitination of PTEN which acted to stabilise the protein and reduce proteasomal degradation. This in turn diminished *MSR1* mRNA and protein levels (100). Conversely, stimulation with TNF-α or IL-6 increased the accumulation of oxidised-LDL *in vitro* by inducing *MSR1* expression *via* the NF-κB pathway (101). *MSR1* expression can also be induced by angiopoietin-like protein 8 (ANGPTL8), a hormone linked to the regulation of lipid metabolism and the development of atherosclerosis. Overexpression of ANGPTL8 significantly promoted foam cell formation *via* increased cholesterol uptake mediated partially by MSR1 upregulation. However, the exact mechanism behind this upregulation was not determined (102). This highlights again that MSR1 can be attributed to both the pathophysiology of and protection against atherosclerosis.

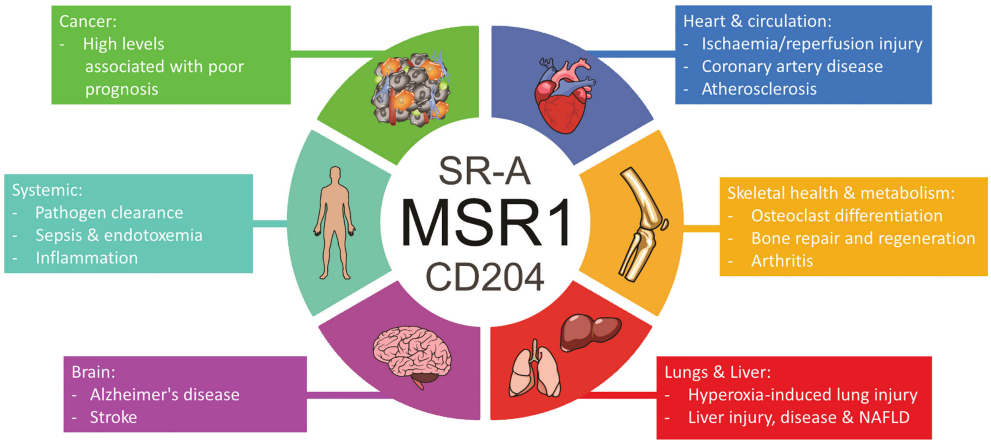

FIGURE 8
Overview of the involvement of MSR1 in health and disease states.

Myocardial infarction and ischemia/reperfusion injury

Inflammatory response is an important phase after myocardial infarction and ischemia/reperfusion (I/R) injury. Alternatively activated (M2) macrophages could have enhanced protective effects, they have potential as anti-inflammatory cells, which can reduce immune responses and prevent autoimmune pathology. MSR1, ITGA4, and CYBB have been shown to be up-regulated in both MI and I/R injury (103). Furthermore, MSR1 induced protection by limiting macrophage polarization towards the pro-inflammatory M1 phenotype and therefore decreasing the secretion of IL-1β, IL-6, TNF-α, and MMP9. This aids in the remodelling of the infarct which in turn protects against potentially lethal cardiac rupture. The risk of cardiac rupture with myocardial infarction was increased after targeted knockout of the MSR1 receptor. This resulted in increased activation of the ASK1/p38/NF-κB signalling pathway which mediates apoptosis, thus leading to further myocardial deterioration (104, 105). However, mice lacking MSR1 had a significantly smaller infarct size and better cardiac function following injury in an independent study. This was due to attenuation of p53 mediated apoptosis and, contrary to previous findings, loss of NF-κB signalling. In this scenario, apoptosis was reduced due to increased expression of miR-125b in Msr1-/- macrophages upon myocardial I/R injury and hypoxia/reoxygenation-induced cell damage (106). Whilst the experimental models used here were different in their methodology, they are modelling the same pathophysiologic process, therefore the striking contrast is surprising. The main difference between the two models used is the time spent ischaemic, so perhaps the temporal differences influenced the vastly different signalling consequences observed in Msr1-/- mice.

Alzheimer's disease

The microglia, specialised tissue macrophages, of the central nervous system maintain vital processes such as neurogenesis and synaptogenesis, as well as control immune processes in the brain (107). They have been shown to bind and phagocytose β-amyloid fibrils *via* MSR1. This in turn has a rather dichotomous effect. This helps to prevent the formation of the neurotoxic amyloid plaques that contribute towards the development of Alzheimer's disease. However, parallel to this is the activation of microglia resulting in increased nitric oxide and reactive oxygen species (ROS) production, which can result in damaging neuroinflammation (21, 22). Adding further to the positive effects of MSR1 expression in Alzheimer's disease, MSR1 expression is reduced in the ageing brain concomitantly with increased β-amyloid deposition and reduced working memory capacity. This results in increased mortality and secretion of proinflammatory molecules (ROS, IL-1β, and TNF-α), and decreased release of anti-inflammatory cytokines (IL-10 and TGF-β) (108). These studies imply that pharmacological enhancement of MSR1 activity or expression may be of potential therapeutic benefit in the treatment or prevention of Alzheimer's disease. One potential method of enhancing MSR1 activity to aid the clearance of β-amyloid was highlighted during the development of a novel early diagnostic tool for Alzheimer's disease. Superparamagnetic iron oxide nanoparticles (SPIONs) conjugated to a β-amyloid oligomer specific antibody (W20) and a neuroprotective heptapeptide (XD4) were able to promote the phagocytosis of β-amyloid *via* MSR1 (109). However, apolipoproteins A-I and E have been shown to adhere to MSR1. These apolipoproteins are present in Alzheimer's plaques and interaction with MSR1 causes macrophage retention, thus leading to increased inflammation in areas

already at risk from neurological damage (18). Therefore, therapeutic intervention by the modulation of MSR1 activity should be done with this activity in consideration. However, as microglia from MSR1 knockout mice only showed a 60% decrease in β-amyloid clearance, other scavenger receptors may also be responsible for some of the clearance and signalling changes (110).

Cerebral reperfusion injury

MSR1 has also been shown to contribute to cerebral reperfusion injury (i.e., stroke) by polarizing macrophages towards the M1 inflammatory phenotype, activity contradicted by later findings in hepatic inflammation (4). This causes inflammatory damage to ischaemic areas and results in a significantly increased infarct size. MSR1 deficiency also attenuated NF-κB activity and apoptotic signalling, both of which normally contribute to ischaemic injury (111, 112). However, contradictory findings have shown that loss of MSR1 leads to reduced clearance of damaged associated molecular patterns (DAMPs), such as HMGB1, more severe inflammation and therefore increased neuronal injury in murine ischaemic stroke (113). This finding was corroborated through the use of a rat model of middle cerebral artery occlusion. Here, enhancement of MSR1-mediated DAMP clearance significantly reduced infarct size and ameliorated neurological deficits. The activity of MSR1 was heightened through the use of a phthalide derivative which induces the MAFB-MSR1 pathway, MAFB being a transcriptional regulator of MSR1 expression (114). This contradiction again mirrors the apparent dualistic nature of MSR1 seen in other disease states, but the use of similar disease models offers no indication as to what drives these differences.

Skeletal health and bone metabolism

Macrophages have the ability to fuse with other macrophages to form multinucleated giant cells. Commonly viewed as the resident macrophages of bone, osteoclasts are a form of multinucleated giant cell associated with bone repair and regeneration, due to their unique ability to resorb bone (107). MSR1 mediates osteoclast differentiation, potentially by facilitating interaction between osteoclast precursors and osteoblasts, and therefore has a role in normal bone metabolism. Loss of MSR1 decreased osteoclast populations, increasing bone density due to reduced resorption (115). Further to this, involvement of MSR1 has been demonstrated in bone regeneration after fracture. Msr1-/- mice displayed delayed intramembranous ossification, a key step of bone repair where mesenchymal stem cells differentiate into osteoblasts which then deposit mineralized extracellular matrix. This is potentially the result of a reduction in MSR1-mediated osteogenic differentiation of bone marrow stem cells. Moreover, MSR1-mediated promotion of bone repair is controlled through the PI3K/AKT/GSK3β/β-catenin pathway which induces the production of several osteoprotective factors. However, loss of MSR1 inactivates this pathway and subsequently impedes mitochondrial oxidative phosphorylation and M2 polarization (116). Mechanistically, it is unclear exactly how MSR1 modulates this signalling pathway, and therefore requires further investigation.

Lung injury

Alveolar macrophages serve to phagocytose inhaled particles and respiratory pathogens, therefore MSR1 holds several key protective functions here, owing to its scavenging capabilities. The receptor has been shown to diminish hyperoxia-induced lung injury by limiting macrophage activation thus leading to significantly lower expression of iNOS and slowed generation of TNF-α (117). Alveolar macrophages also limit pulmonary inflammation, in chronic obstructive pulmonary disease and asthma, after oxidant inhalation by utilising MSR1 to scavenge proinflammatory oxidised lipids (118). Furthermore, MSR1 expressed on lung DCs has been shown to control specific immune pulmonary responses to inhaled particles and pathogens by reducing DC migration towards lymph nodes. This therefore mitigates unwanted immune responses to common aeroallergens (119). Not only are MSR1 levels linked to lung injury, but also the MSR1-coding SNP P275A in macrophages was associated with susceptibility to chronic obstructive lung disease in smokers (120).

Liver injury and disease

Macrophages in the liver, called Kupffer cells, mediate immune response and hepatic tissue remodelling (107). Chronic inflammation or infection in the liver can result in fibrosis, cirrhosis, and hepatocellular carcinoma. Due to the role of MSR1 in lipid uptake, MSR1 deficiency can reduce hepatic inflammation and changes in hepatic lipid metabolism in mice when fed with a high-fat diet (76). However, MSR1 expression is crucial for promoting M2-macrophage activation and polarisation during hepatic inflammation. Expression of MSR1 increases in the later stages of hepatotropic viral infection, shifting the phenotype of non-tissue resident macrophages towards the M2 phenotype. Indeed, the loss of M2-like features was more pronounced in cell lines lacking expression of MSR1 when compared to WT mice. This reveals the importance of MSR1 in the polarisation of macrophages to M2. This phenotype is important in the liver as it is linked

with tissue repair which prevents the deposition of fibrotic tissue. MSR1 interacts with MERTK (Tyrosine-protein kinase Mer), MERTK activation may then inhibit the mTOR pathway which acts to modulate macrophage polarization. MSR1 knockdown cells exhibited enhanced mTOR phosphorylation (4). Soluble anti-MSR1 blocked two known key early events during apoptotic cell uptake: the sequential tyrosine phosphorylation of MERTK and of PLCγ2 (20). These data support the MSR1/MERTK complex as a potential target to manipulate apoptotic cell clearance and hence, resolution of inflammation, and infections.

Recent work by our group showed that MSR1 is directly responsible for saturated fatty acids uptake in Kupffer cells and foamy macrophages, leading to an inflammatory response independent of TLR4 in non-alcoholic fatty liver disease (NAFLD). Foamy macrophage generation and fibrosis was also impeded in mice lacking MSR1, indicating potential therapeutic benefit in non-alcoholic fatty liver disease (NAFLD) (76) and other inflammatory diseases, such as atherosclerosis, where foam cells contribute to pathogenesis (121). MSR1 is also upregulated on the cell surface, as well as in its soluble form, in the livers of patients with viral hepatitis infection (122), autoimmune hepatitis disease and in mice subjected to concanavalin A-induced hepatitis (123). Soluble MSR1 binds directly to activated T cells and has an inhibitory effect, potentially *via* an IL-2 dependent mechanism, acting as a negative regulator of CD8+ T-cell activation and expansion. This has a protective effect by limiting hypersensitivity and activation of T-cells. Genetic ablation of MSR1 resulted in a higher sensitivity to concanavalin A-induced liver injury, coinciding with excessive levels of IFN-γ production and STAT1 phosphorylation (123). Homeostatic regulation of T cell activity, mediated by MSR1, becomes especially important in autoimmune hepatitis.

Response to pathogens

Macrophages are a vital component of the innate immune system and participate heavily in host defence. Therefore, it is no surprise that bacterial and viral components are featured amongst the wide range of ligands that interact with MSR1. MSR1 can bind and clear free bacterial components such as LTA (40) or LPS (39) but can also bind bacteria directly. The receptor has been shown to bind and protect against different gram-positive bacteria: Streptococcus pyogenes (40), S. agalactiae (40), S. pneumoniae (40, 124), Staphylococcus aureus (40, 125), Enterococcus hirae (40), and Listeria monocytogenes (40); as well as gram-negative bacteria: Neisseria meningitides (126), Escherichia coli (127) and Francisella tularensis (128). Whilst mostly host protective, MSR1 has been described to be a negative regulator of multinucleated giant cell formation in tuberculosis. These cells protect the host against Mycobacterium tuberculosis infection by improving antigen presentation and mycobacteria killing (129). MSR1 can differentially regulate bacteria-induced inflammatory response *via* several methods. First by limiting the cell-surface availability of ligands, such as LPS, *via* its scavenging ability. Thus, dampening the TLR4 mediated response. Conversely, the receptor can act as a co-receptor by binding ligands such as trehalose dimycolate, increasing the likelihood of interaction with TLR2. Furthermore, endocytosis by MSR1 enhances the intracellular availability of ligands, allowing interaction with TLRs and nod-like receptors (NLRs) at the endosome (41).

The function of MSR1 in host protection against viruses is varied. The receptor directly binds to, internalises and degrades adenovirus type 5 (130) and protects against herpes simplex virus type-1 (96). MSR1 is also required for the sensing of human cytomegalovirus through interaction with TLR3 and TLR9 (38). Furthermore, it acts as a critical component of TLR3-mediated response to hepatitis C infection by endocytosis of dsRNA (34). The receptor may also mediate autophagy, contributing to the innate response against Chikungunya virus infection (131). Again, the activity of MSR1 in this area is not always protective. The receptor has been shown to contribute towards the pathogenesis of virus-induced fulminant hepatitis by enhancing neutrophil NETosis, a unique form of cell death, and subsequent complement activation (132). Furthermore, MSR1 has been implicated in the pathogenesis of vesicular stomatitis virus (VSV) infection. Mice lacking MSR1 displayed significantly decreased mortality and morbidity, with MSR1 being shown to have a significant impact on VSV infection in the central nervous system. Cellular entry of VSV was dependent on MSR1 functioning as a co-receptor for low density lipoprotein receptor (LDLR) mediated uptake (133).

Recent evidence has emerged indicating a potential role for MSR1 in SARS-CoV-2 infection, however the limited data accrued so far is already contradictory. COVID-19 patients with severe symptoms were found to have significantly increased MSR1 expression on circulating monocytes and DCs (134). M2 macrophages infected with SARS-CoV-2 also showed increased MSR1 expression after 48 hours (135). However, in a separate study, the spike protein of SARS-CoV-2 was shown to reduce the expression of MSR1 on macrophages *via* interaction with DDX5, a protein involved in pre-mRNA splicing (136). Blood transcriptome data gathered from patients with severe COVID also showed decreased MSR1 expression (137). Ultimately, the regulation of MSR1 expression and resulting signalling consequences in SARS-CoV-2 infection are currently unknown.

The molecular mechanism behind MSR1 mediated TLR4 signalling inhibition was the first endocytosis and phagocytosis independent MSR1 signalling mechanism to be described. This mechanism acts as a fine control for LPS-induced TLR4-NF-κB signalling. MSR1 regulates this pathway by directly interacting with the TRAF-C domain of TRAF6, this prevents its dimerization or ubiquitylation which is normally required for TLR4-mediated activation. This prevents TRAF6 from activating IκB kinase and the MAPK cascade. The resulting sequestration of NF-κB prevents the activation of target genes

involved in immunity and inflammation and ultimately dampens the adaptive immune response driven by TLR4 activation (138).

Endotoxemia and sepsis

Further to its role in bacterial immune response, MSR1 has a complex and contradictory role in endotoxemia or systemic inflammation, often used as a model substitute for sepsis despite clear disparities and limitations in their nature (139). Several studies indicate that MSR1 ameliorates sepsis by suppressing the pro-inflammatory response, in particular by reducing TNF-α signalling and TLR4-induced activation of NF-κB, potentially by limiting the availability of free LPS (140–144). Further to this, selective and transient depletion of Msr1 in macrophages *in vivo* resulted in elevated serum concentrations of TNF-α and IL-6 as well as decreased survival rate when mice were challenged with LPS-induced endotoxemia (145). On the contrary, MSR1 has been shown to enhance the production of TNF-α in LPS-treated J774A.1 macrophages (146). This finding was recapitulated in a separate study, however the inclusion of fucoidan alongside LPS treatment may account for the increased pro-inflammatory signalling observed (29, 147). Further contradicting the protective nature of MSR1, LPS treatment resulted in a higher mortality in wild type mice than in Msr1-/- (148). MSR1 played a clear detrimental role in the disease pathophysiology of the cecal ligation and puncture model of sepsis, enhancing pro-inflammatory signalling through interaction with TLR (149, 150). Further to this, blockade of MSR1 by berberine alkaloids restricted the receptors' ability to endocytose LPS. This prevented downstream activation of the caspase-11 pathway which normally acts to induce endotoxin-mediated coagulation syndrome, often seen in bacterial sepsis (31).

The contradiction in findings here, as in atherosclerosis, offers no concrete understanding of the molecular mechanisms. Moreover, the inclusion of fucoidan when investigating pro-inflammatory response may confound results as it has since been shown to induce JNK signalling. Furthermore, differences in expression or structure of MSR1 may, as evidenced between C57BL/6J and A/J mice, lead to differences in inflammatory response (142). LPS is known to activate TLR4 and induce the expression of pro-inflammatory cytokines through the MAPK kinase and NF-kB pathway (151). Therefore, the controversial role of MSR1 in sepsis could be masked by the activation of other receptors by LPS.

Rheumatoid arthritis and osteoarthritis

MSR1 has been investigated for its use as a biomarker and the pathological role it plays in rheumatoid arthritis (RA), a highly debilitating chronic autoimmune disease. Early detection is dependent on sensitive and specific biomarkers. At current the two clinically used biomarkers, rheumatoid factor and anti-cyclic citrullinated peptide antibody (anti-CCP), only show moderate discriminatory ability. A large-scale multicentre study revealed that soluble MSR1 could be utilised as a diagnostic marker for RA, offering competitive sensitivity and specificity values. Further to its diagnostic value, the role of MSR1 in RA pathogenesis was correlated with disease severity in two different models: 1) the administration of soluble MSR1 which increased disease severity, and 2) inhibition or genetic ablation of MSR1 which reduced disease severity. MSR1 mediated disease progression was concomitant with increased T cell activation. Resistance to RA in Msr1-/- mice was associated with decreased IL-17a and TNF-α production by T helper cells (152).

In osteoarthritis (OA), macrophages exacerbate cartilage damage through the production of pro-inflammatory cytokines. Macrophage activation is a key step in OA initiation along with upregulation of other scavenger receptors, and the release of polyanionic molecules. Utilising dextran sulphate nanoparticles to encompass triamcinolone acetonide, a corticosteroid used in OA treatment, MSR1 was directly targeted to reduce the viability of macrophages. This in turn diminished pro-inflammatory cytokine mediated cartilage damage (153). Similarly, MSR1 can be targeted for the treatment of RA using methotrexate in place of triamcinolone acetonide (154, 155). These studies show that, whilst MSR1 is upregulated and implicated across the spectrum of disease, the ligand binding and endocytic capabilities of the receptor can be exploited to deliver disease modifying drugs specifically to macrophages.

MSR1-positive tumour associated macrophages in cancer

Tumour associated macrophages (TAMs) can be held partially responsible for the propagation of tumours through their action on several of the hallmarks of cancer: avoidance of immune destruction, activating invasion and metastasis, or even in angiogenesis (Figure 9) (156–160). Macrophage phenotype becomes shifted towards the immunosuppressive M2-like subset by tumour-derived cytokines such as IL-4, IL-10, and IL-13. TAMs are able to inhibit the action of cytotoxic T-cells *via* IL-10 secretion and support regulatory T-cells, thus leading to immune evasion and tumour proliferation (157). Following this, TAMs secrete pro-migratory factors such as epidermal growth factor (EGF), cysteine cathepsins and matrix metalloproteinases (MMPs). TAMs located at the migratory front of the tumour utilise these matrix-degrading enzymes to aid migration by breaking down the extracellular matrix (ECM) (161, 162). Once migration has been facilitated, the next barrier in benign-

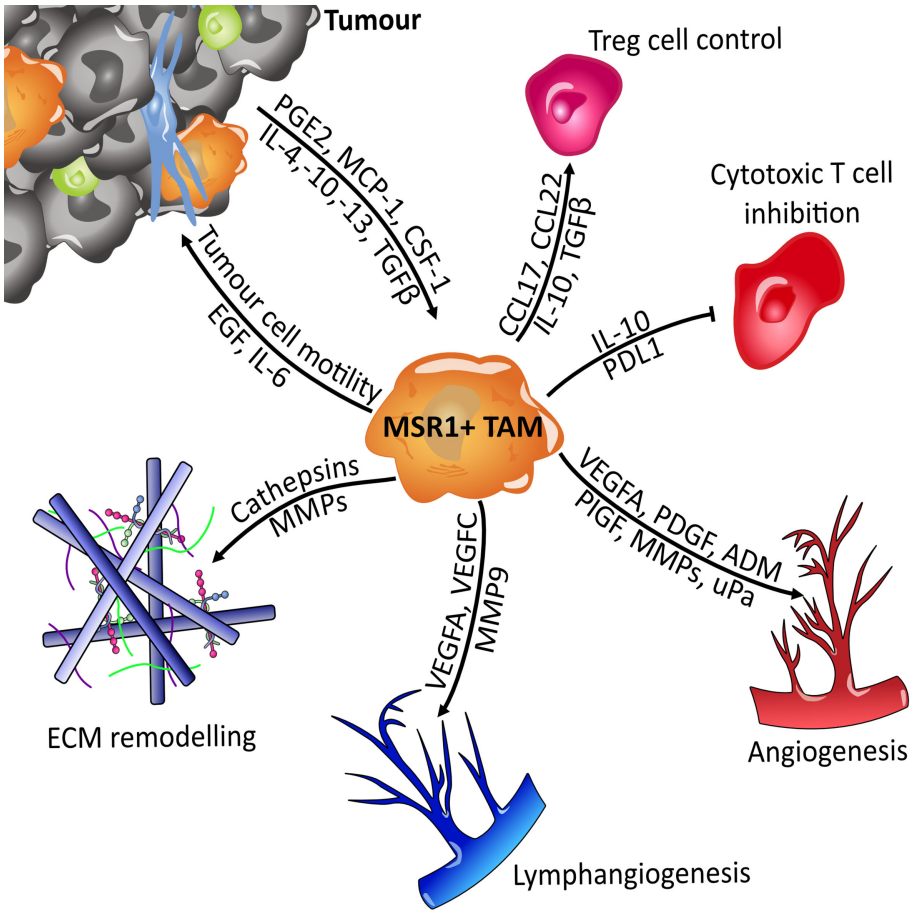

FIGURE 9
Overview of MSR1-positive TAMs signalling. Macrophages become polarised towards the M2-like tumour-associated macrophage (TAM) phenotype by tumour derived factors such as IL-4 and CSF-1. Once polarised they then support tumour expansion and metastasis by secreting factors that influence several of the hallmarks of cancer. They suppress the immune system by regulating the function of T_{reg} (Regulatory T cells) and cytotoxic T cells (CD8+). Metastasis and growth are facilitated through breakdown of the extracellular matrix (ECM), the induction of angiogenesis and lymphangiogenesis, and directly through cytokine mediated influence of tumour cell motility. Adrenomedullin (ADM); colony stimulating factor 1 (CSF-1); epidermal growth factor (EGF); interleukin (IL); matrix metalloproteinases (MMPs); monocyte chemoattractant protein-1 (MCP-1); placental growth factor (PlGF); platelet-derived growth factor (PDGF); programmed death-ligand 1 (PDL1); prostaglandin E1 (PGE-1); transforming growth factor beta (TGFb); urokinase plasminogen activator (uPa); vascular endothelial growth factor A (VEGFA).

to-malignant tumour transition is hypoxia caused by a deficiency in vasculature. Therefore, an angiogenic switch is required to allow neovascularisation and the supply of oxygen to the growing tumour. This switch is provided by TAMs with the secretion of angiogenic factors such as VEGF-A, VEGF-C, and adrenomedullin. Pro-angiogenic cytokines such as uPA are also produced. The MMPs produced by TAMs also have a secondary effect, aiding angiogenesis by allowing the release of extra growth factors from extracellular depots (157, 163). Lymphangiogenesis is also stimulated in a similar fashion to angiogenesis, this further increases the migratory potential of the tumour. It is therefore clear that TAMs have an important role to play in tumorigenesis.

MSR1 is emerging as an important TAM marker, with recent studies linking high expression levels of the receptor to a significantly poor prognosis and increased severity of multiple forms of cancer. However, the exact mechanistic role that MSR1 plays in TAM-mediated tumorigenesis remains elusive.

Whilst deletion of MSR1 and the surrounding chromosome region in tumour cells has been associated with poor cancer prognosis (Figure 4), the expression of MSR1 in tumour cells is ultimately low. Therefore, the correlation between MSR1 expression in tumour cells and carcinogenesis has not been well studied so far. However, there are an abundance of studies that show that MSR1 is an essential marker for TAMs and is associated with cancer progression and poor prognosis. However, the use of one single marker may seem like a reductionist approach, therefore multiplexed immunohistochemistry (IHC) has been developed to allow delineation of specific macrophage

subpopulations within the tumour microenvironment (TME). Several sub-populations can be identified using M1 markers (HLA-DR/CD68), M2 markers (CD163/CD68), pan-macrophage markers (CD68/CK), and MSR1 as a TAM marker (164). It has recently been published that not only is the MSR1 marker relevant in tumour prognosis but also the ratio of T cells, B cells, and MSR1+ TAMs. A high ratio of CD8+ T cells to MSR1+ TAMs indicated a favourable postoperative prognosis in prostate cancer (165). Similar prognostic value was confirmed in thymic carcinoma where the ratio of CD8+ T cells/MSR1+ TAMs and CD20+ B cells/MSR1+ TAMs indicated prognostic effect in the stroma (166).

MSR1+ TAM populations have the potential to be used as an effective prognostic marker for various cancers. A higher number of MSR1+ TAMs were present in the tumour stroma area than in the primary tumour and this was associated with multiple clinicopathological factors, poor prognosis, and shorter survival time in non-small cell lung cancer (NSCLC) (167), lung squamous cell carcinoma (168), lung adenocarcinoma (169, 170), uterine cervical adenocarcinoma (171), invasive ductal carcinoma (172), glioma (173), and muscle-invasive bladder cancer (174). Moreover, MSR1+ TAMs present more levels of IL-10 and MCP-1 which are involved in accumulation, migration, and polarisation of M2 macrophages (168, 169). In addition, MSR1 levels in TAMs are shown to have a positive correlation with the cancer's grade in glioma (175) and colorectal adenoma (176). Furthermore, highly proliferative cells can induce macrophage colony-stimulating factor (M-CSF) which increases MSR1 expression and M2 polarisation in macrophages (175, 177).

TAMs have been associated with the promotion of tumour metastasis. Besides the use of IHC to analyse MSR1+ TAMs, flow cytometry also provides a multiparameter analysis to study cell invasion and tumour metastasis of breast cancer (178), gastric cancer (179), oral squamous cell carcinoma (180), and ovarian cancer (181). The highly complex and immune cell-rich desmoplastic stroma contributes significantly to tumour cell migration and metastasis. Many studies have indicated that tumour cells can promote M2 polarisation of TAMs inducing the upregulation of chemokines, cytokines, and matrix metalloproteinases associated with tumour promotion, such as: TNF-α, MMP-1,-2,-7,-9,-14, VEGF-B,-C, and CSF-1 (179, 182, 183). In pancreatic cancer, MSR1+ macrophages were found within the TME and at the migratory front of the cancer which was associated with tumour aggressiveness (184). Comparison studies of brain metastases and primary lung tumours have indicated an increased number of MSR1+ TAMs in the TME of these metastases, but not from other cells of the immune system (185). Furthermore, infiltration of MSR1-positive macrophages into the TME of colorectal cancer was connected to poor prognosis due to increased proliferation and invasion of tumour cells (186). A significant link between the number of MSR1+ macrophages and lymphangiogenesis was also observed in pancreatic ductal adenocarcinoma thus providing insight into how these tumour-promoting macrophages facilitate metastasis (184). However, this association has been contradicted by recent weighted gene co-expression network analysis (WGCNA) of immune infiltration cells in osteosarcoma metastasis. This study highlighted that macrophage infiltration was decreased in these metastases and MSR1 was identified as having an anti-metastatic role and linked to an increase in overall survival (187). Although, as this analysis was carried out using transcriptome data, these findings need experimentally corroborating through the use of other omics methods.

Whilst the relationship between MSR1 and cancer has mainly been represented as a correlation between increased expression and poor prognosis, there is also evidence that indicates isoform driven effects in cancer. Isoforms of MSR1 were differentially expressed in primary melanomas and benign melanocytic nevi. One isoform, more prevalent in melanoma, was characterised by a gain of collagen and SRCR domains. As the collagen-like domain is the site for ligand binding, this may indicate that cancer derived ligands work *via* MSR1 to help drive the formation of the metastatic form of the disease. On the other hand, the most upregulated isoform in benign nevi was a non-coding transcript with loss of an open reading frame (188). Further suggesting a role for MSR1 in benign to malignant transformation of tumours. The clinical studies outlined above all relied on IHC analysis to investigate the potential for MSR1 positive TAMs to be used as a prognostic marker in several tumours. Whilst these investigations provide valuable insight into the link between these TAMs and aggressiveness of the tumour, they offer limited functional or mechanistic insight into the pathophysiologic activity of MSR1 in the TME. Mechanistic insight into this relationship was explored by culturing human monocyte-derived macrophages with conditioned media from three different breast cancer cell lines. Conditioned media was shown to stimulate MSR1 over-expression but not CD163 or CD206, two typical markers for M2 macrophages. Thus, showing that the increased expression of MSR1 is a result of a more specific reprogramming rather than a simple M2-like switch (172). Proteomic analyses have attempted to discover the tumour cell ligands that could activate MSR1 in TAMs (189). Unfortunately, the cancer cell derived molecules responsible for MSR1 upregulation are yet to be identified. A potential candidate, however, is hyaluronan expression of which correlates highly with the number of M2 macrophages in the TME of malignant breast tissues. Furthermore, inhibition of hyaluronan synthesis or its receptor, CD44, significantly reduced M2 polarisation and MSR1 expression (190). This gives one possible explanation as to how MSR1 becomes significantly upregulated in various cancers. More interestingly, hyaluronan is an anionic biopolymer, therefore it might act on and signal through MSR1 directly within the TME (191). This is yet to be demonstrated experimentally.

Taken together, there is striking evidence that MSR1 is a valuable prognostic marker for the severity of multiple types of cancer. Furthermore, there is mounting evidence that MSR1

itself holds some pro-tumour activity with targetable signalling pathways remaining elusive. However, the high expression of MSR1 in the TME, coupled with its ligand binding capabilities, make it a promising candidate for pharmacologic intervention.

Therapeutic strategies

The first approach was to use the MSR1 ligands themselves, which can potentially competitively inhibit its function. Fucoidan, poly I, and poly G were able to inhibit tumour progression and invasion *via* MSR1 in ovarian and pancreatic cancer models (182). However, the caveat to consider here is that ligands will not act solely as inhibitors, as different ligands will often activate different inflammatory signalling pathways. Further therapeutic potential was identified by exploiting the ligand binding capabilities of MSR1 to generate a highly selective conjugate for TAM-targeted photodynamic therapy (PDT). Polyanionic sodium alginate was conjugated to phthalocyanine, a clinical photosensitiser, to target MSR1 and accumulate in TAM-rich areas. The sodium alginate-phthalocyanine conjugate achieved an 87% tumour inhibition growth rate. Coupled with low toxicity to normal tissue, these results show promise in targeting TAMs *via* MSR1 to reduce their effect on tumour growth and metastasis (192). It is clear that MSR1 acts within the TME, however a defined tumour-specific ligand and signalling cascade are yet to be clarified, this therefore requires further investigation.

To date, no specific inhibitors for the MSR1 receptor have been described. However, a small 16-amino acid amphipathic peptide, 4F, inhibited MSR1 and drastically reduced the invasion of ovarian and pancreatic cancer cells, and reduced tumour growth *in vivo* (189). Furthermore, tumours with MSR1 mutations increased sensitivity to treatment with the AKT inhibitor GSK690693, according to the Genomics of Drug Sensitivity in Cancer (GDSC) project using the Pan-cancer database (193).

Adding further complexity to the effects of MSR1 in cancer, upregulation of the receptor is not always associated with poor prognosis. Irradiated mice which received transplanted bone marrow from Msr1−/− mice developed chronic myeloid leukaemia (CML) significantly faster than those which received WT cells. Furthermore, ectopic overexpression of MSR1 delayed CML development. The tumour promoting effect of loss of MSR1 was determined to be a result of activation of the PI3K-AKT-GSK3β pathway and increased expression of β-Catenin. This indicates that enhancement of MSR1 function may be a novel therapeutic intervention for CML (194).

Vascular leukocytes (VLCs) are similar to TAMs as they are recruited to the tumour and phenotypically switch to a tumour-promoting population. MSR1 was also found to be expressed in the VLCs in ovarian cancer. Therefore, an antibody-based method was developed to deplete VLCs from the peritoneum, using saporin toxin (ZAP) conjugated to the 2F8 anti–SR-A monoclonal antibody. This immunotoxin was able to specifically deplete the VLC population and reduce tumour burden (195). In another context of disease, therapeutic inhibition of MSR1, *via* monoclonal antibody treatment, decreased the release of TNF-α both in an NAFLD mouse model and in ex vivo human liver (76).

MSR1 expression significantly correlated with immune checkpoints (173). Suggesting that MSR1 positive TAMs synergise with immune checkpoint regulators to inhibit the activity of T cells in the TME. This can promote tumour progression and limit the efficacy of therapeutic interventions designed to prime the adaptive immune system. MSR1 is known to bind and internalise multiple heat shock proteins (HSP) such as HSP27 (25), HSP110 and GRP170 (26), and GP96 and GRP94 (27). Interaction of MSR1 with HSPs subsequently induces an immunosuppressive response by dampening TLR4, NFkB and MAP kinase activity (196). The lack of MSR1 significantly enhanced HSP- or LPS-mediated vaccine activity against poorly immunogenic tumours (197, 198). HSP-adjuvant vaccines function on the basis that TLR4 signalling can be targeted to lead to the stimulation of a heightened immune response against tumour-associated antigens. Therefore, the finding that loss of MSR1 further enhances this immune response indicates cross talk between MSR1 and TLR4 signalling. This increase in antitumour response could be attributed in part to an enhanced CD8+ T cell response (197). MSR1 may act to hinder the efficacy of therapeutics designed to exploit the adaptive immune system. It was further demonstrated that MSR1 inhibition or deletion improved the ability of DCs to generate antitumour responses to melanoma, improving the expansion and activation of CD8+ T cells specific for melanoma antigens. Adding further depth to these findings, the use of Msr1-/- DCs to produce antigen-targeted vaccines resulted in increased infiltration of not only CD8+ T cells but also natural killer (NK) cells as well as increased intratumoural ratios of both CD4+ and CD8+ T effector cells to CD4+CD25+ T regulatory cells, all of which contribute to the elimination of malignant cells (199). Furthermore, MSR1 has been shown to impair the cytotoxic antitumour response of NK cells post-surgery. This impairment was linked to an increased expression of MSR1 on NK cells following surgery, resulting in increased lipid accumulation. Lipid-laden NK cells subsequently showed a decrease in expression of important NK cell receptors and decrease in the ability to lyse tumours (200).

The immunomodulatory effects of MSR1 were also confirmed during radiation therapy for prostate cancer. Combination of radio and immunotherapy is beneficial in local tumour control as irradiation results in tumour-specific antigen shedding. These antigens can then be processed by antigen presenting cells such as DCs, ultimately resulting in an anti-tumour immune response. *In situ* vaccination with DCs in which MSR1 had been downregulated, alongside ionizing

radiation, significantly suppressed the growth of murine prostate cancer and a reduction in distant metastases was also seen. Recapitulating earlier findings, a significant increase in tumour infiltrating CD8+ T cells was identified (201).

Discussion

In this review, we have explored the physiological and pathophysiological importance of MSR1 in different tissues (Figure 10), and more specifically, the changes influenced by post-translation modifications, differential expression, mutation, or ligand binding, which may have consequences for MSR1 activity and its function in macrophages. MSR1 holds canonical physiological roles mediating endocytosis of modified lipids, phagocytosis of pathogens and apoptotic cells, cell adhesion, and cytokine production. However, more recently, MSR1 has been implicated in various signalosomes that trigger inflammatory and tumorigenic pathways. The ability of MSR1 to either ameliorate or potentiate disease is, in part, a result of the wide ligand binding capabilities of MSR1, giving rise to multiple different signalling pathways. Contradictory findings of MSR1 activity in diseases such as atherosclerosis and sepsis may also potentially indicate a much more complex mechanism behind the activity of MSR1. The intrigue here, however, stems from the fact that MSR1 holds no distinct intracellular signalling motif. This leads to one of the widest gaps in the knowledge surrounding the signalling of MSR1, with only one full signalling pathway identified so far. This being ubiquitin chain-mediated recruitment of the TAK1 complex on the phagosome. Identification of binding partners, such as HSP90, or post-translational modifications, such as ubiquitin chain linkages or phosphorylation, that facilitate MSR1 signalling changes will bridge this gap and offer a greater insight into how exactly MSR1 can influence such a range of processes.

Furthermore, the signalling pathways outlined thus far all have pro-inflammatory consequences. This is interesting as MSR1 is most often correlated with the anti-inflammatory M2 macrophage phenotype, especially in the TME. Therefore, investigations into anti-inflammatory signalling downstream of MSR1 are vital to fully understand the influence of MSR1 expression in inflammatory disease. Manipulating M2 macrophages through MSR1 may represent a new targeted therapeutic approach for diseases such as cancer, arthritis, and other inflammatory diseases. Proving that

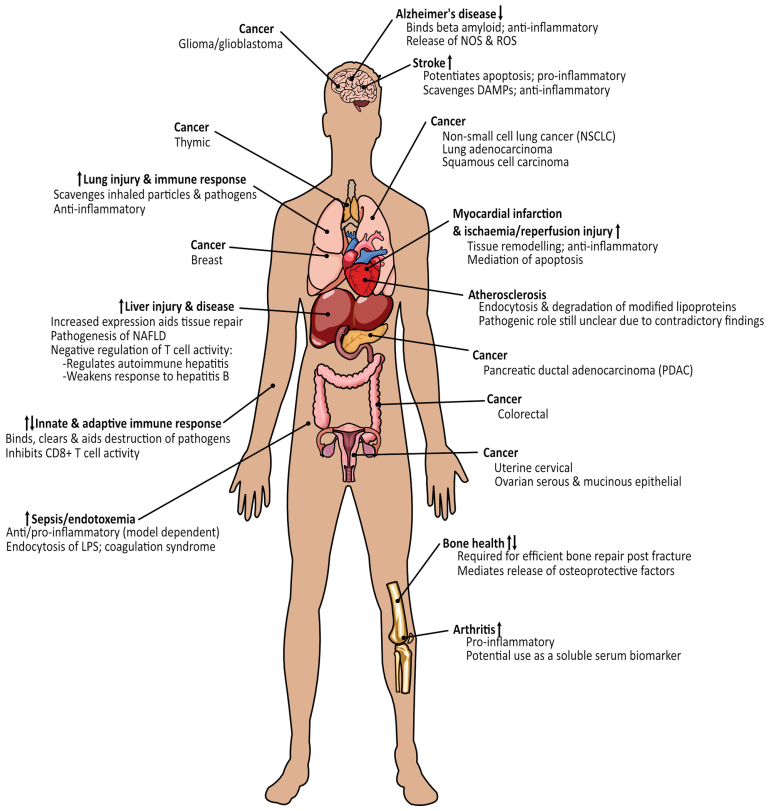

FIGURE 10
Overview of the processes and diseases where MSR1 holds either a protective or damaging function. Arrows indicate whether expression of MSR1 is increased or decreased.

targeting cells that promote disease progression, rather than the disease itself, is a viable therapeutic option and that MSR1 is an ideal receptor to do so due to its wide ligand-binding capabilities and endocytic function. However, one caveat that is often overlooked in MSR1 analyses is the use of fucoidan, or other MSR1 ligands, as inhibitors. As is clear from the pro-inflammatory pathways previously described, fucoidan does not act as an inhibitor of MSR1, in fact it is very much the opposite, activating multiple different pathways. Therefore, studies which utilise fucoidan to block the activity of MSR1, or other ligand binding to MSR1, must take this into account when interpreting results as fucoidan may partly re-polarise M2 macrophages towards an inflammatory phenotype. The use of ligands as inhibitors may account for some of the differences seen between experimental models. Differences may also be seen due to the plastic nature of macrophages influencing the availability of signalling molecules, co-receptors, or post-translational machinery, as it is clear that MSR1 relies on these to stimulate signalling cascades.

Overall, it is evident that MSR1 plays a dual role in a multitude of difference processes. However, there is still a long way to go before the receptor is fully understood. A full understanding may be accomplished through deeper interrogation of MSR1 signalling. This is especially important to understand its role in cancer, where detailed molecular mechanisms are absent despite a wealth of data indicating its role as a prognostic marker for disease severity. Further to its activity on macrophages, the influence of MSR1 on other adaptive immune cells such as B cells, T cells and NK cells is also largely unknown. Determining how MSR1 communicates with other cell types may also reveal how MSR1 influences such a wide variety of processes. Pharmacologic modulation of MSR1 activity in disease may also better indicate the true role of the receptor. Further insights in the future will likely shed light on the molecular details of MSR1 functions, thus clarifying its clinical value for each inflammatory pathology and cancer.

Author contributions

JG planned the concept and design of the review. JG and JM-R collected previous literature on the topic and drafted the article. JG made the figures. MT performed critical revision of the article. JM-R and MT edited the final version of the article. All authors contributed to the article and approved the submitted version.

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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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Resolution therapy: Harnessing efferocytic macrophages to trigger the resolution of inflammation

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Several chronic inflammatory diseases are associated with non-resolving inflammation. Conventional anti-inflammatory drugs fail to completely cure these diseases. Resolution pharmacology is a new therapeutic approach based on the use of pro-resolving mediators that accelerate the resolution phase of inflammation by targeting the productive phase of inflammation. Indeed, pro-resolving mediators prevent leukocyte recruitment and induce apoptosis of accumulated leukocytes. This approach is now called resolution therapy with the introduction of complex biological drugs and cell-based therapies. The main objective of resolution therapy is to specifically reduce the duration of the resolution phase to accelerate the return to homeostasis. Under physiological conditions, macrophages play a critical role in the resolution of inflammation. Indeed, after the removal of apoptotic cells (a process called efferocytosis), macrophages display anti-inflammatory reprogramming and subsequently secrete multiple pro-resolving factors. These factors can be used as resolution therapy. Here, we review the different mechanisms leading to anti-inflammatory reprogramming of macrophages after efferocytosis and the pro-resolving factors released by these efferocytic macrophages. We classify these mechanisms in three different categories: macrophage reprogramming induced by apoptotic cell-derived factors, by molecules expressed by apoptotic cells (*i.e.*, "eat-me" signals), and induced by the digestion of apoptotic cell-derived materials. We also evoke that macrophage reprogramming may result from cooperative mechanisms, for instance, implicating the apoptotic cell-induced microenvironment (including cellular metabolites, specific cytokines or immune cells). Then, we describe a new drug candidate belonging to this resolution therapy. This candidate, called SuperMApo, corresponds to the secretome of efferocytic macrophages. We discuss its production, the pro-resolving factors present in this drug, as well as the results obtained in experimental models of chronic (e.g., arthritis, colitis)

and acute (e.g., peritonitis or xenogeneic graft-*versus*-host disease) inflammatory diseases.

KEYWORDS

macrophage, apoptotic cells, efferocytosis, resolution of inflammation, inflammation, TGF-β, non-resolving inflammation, macrophage reprogramming

1 Introduction

Inflammation is a natural protective response to fight against any aggression, such as infections. Under physiological conditions, the resolution phase of inflammation allows the body to stop inflammation, and promotes tissue repair to return to homeostasis. Carl Nathan and Aihao Ding were the first to propose the concept of non-resolving inflammation (1). Thus, alterations in the resolution phase of inflammation lead to uncontrolled chronic inflammation responsible for tissue damage. This non-resolving inflammation is encountered in several chronic inflammatory diseases, including atherosclerosis, asthma, inflammatory bowel diseases (IBD), multiple sclerosis (MS), rheumatoid arthritis (RA), as well as cancer (1). Although these chronic inflammatory disorders result from various pathogenic mechanisms, they share this non-resolving inflammation (1). Nevertheless, these chronic diseases are not always controlled by current treatments and development of new therapeutic approaches is urgently required (as recently discussed in an editorial on inflammatory rheumatic diseases (2)).

Resolution pharmacology is a new therapeutic approach based on the use of resolution mediators (3–5). The idea is to stimulate the resolution phase in order to accelerate (or achieve) the return to homeostasis. Indeed, a delay in the resolution can extend the duration of the pro-inflammatory response resulting in tissue damage, which in turn, prolongs the inflammatory state. Among the resolution mediators used in resolution pharmacology, one may evoke specialized pro-resolving lipid mediators (SPM, including lipoxins, resolvins, protectins, and maresins) (6, 7), or proteins (e.g., Annexin-A1 (8), DEL-1 (9) (for reviews please see (5, 10, 11)). Recently, the list of the candidates has been extended to complex biological drugs (e.g., secretomes released by particular cells) or cell-based therapies. This is why the terms "resolution therapy" or "resolution therapeutics" (12) are preferable to describe this therapeutic arsenal able to stimulate the resolution of inflammation.

A critical step in the switch from the initiation/onset phase to the resolution one is mediated by macrophages performing the elimination of apoptotic neutrophils (13–15). This process is called efferocytosis. Efferocytosis enables macrophages to shift from a pro-inflammatory to a pro-resolving function (16–18). This shift, named macrophage reprogramming, consists of the decreased synthesis of pro-inflammatory factors (e.g. IL-1β, IL-12 or TNF) (19, 20) associated with the concomitant increased production of anti-inflammatory mediators (e.g. TGF-β, prostaglandin-E2 [PGE-2] (19). Most of the resolution mediators are produced by pro-resolving macrophages (17, 18). In the current review, we want to focus on these pro-resolving factors released by macrophages after efferocytosis. Before that, we propose to evoke the mechanisms triggered by efferocytosis that stimulate macrophage reprogramming through a pro-resolving profile. Recently, others (17, 18) and we (21–24) have reviewed the different mechanisms leading to anti-inflammatory macrophage reprogramming after efferocytosis. Here, we will focus on the most recent findings. Then, after this part on macrophage reprogramming and pro-resolving factors generated by these cells after efferocytosis, we will describe and discuss a new drug candidate for resolution therapy developed by our laboratory.

2 Efferocytosis as a critical step triggering the resolution phase of inflammation and the release of pro-resolving factors

Macrophages represent heterogeneous cells with different phenotypes. This heterogeneity is encountered even in a given tissue, at steady state, but also during pathogenic situations. The origin of macrophage may influence this heterogeneity (25). Indeed, macrophages may arise from hematopoietic progenitors during embryogenesis and then become tissue-resident macrophages. These tissue-resident macrophages exhibit a self-renew capacity. Macrophage proliferation is critical for maintaining a tissue-resident macrophage pool and participating in tissue homeostasis or protection (26–28). These tissue-resident macrophages are regularly exposed to apoptotic cells generated during normal cell turnover and this exposure imprints an anti-inflammatory program (29, 30). Macrophages may also differentiate from blood monocytes during inflammation (16, 31). Macrophages may therefore exert both pro- or anti-inflammatory functions. Pro-

inflammatory macrophages play a critical role in the onset phase of inflammation, while anti-inflammatory macrophages are involved in the resolution phase of inflammation. Furthermore, macrophage heterogeneity may also depend on their tissue location (16).

In addition to their heterogeneity, another salient property of these cells is their extreme plasticity (16, 32). Macrophages may exert a huge "spectrum" of functions characterized by an array of different phenotypes (33). The two extreme polarized phenotypes of this continuum were initially called M1 and M2 (34). These terms tend to be abandoned nowadays (35), in particular after the description of this "spectrum" model (33). Nevertheless, this M1/M2 classification (34) can be used when describing macrophages in culture during a well-defined condition of stimulation (e.g., the presence of IL-4). The M1 phenotype characterizes pro-inflammatory macrophages involved in anti-infectious responses, and during the onset phase of inflammation. Cells of this subset are also sometimes called "classically" activated macrophages. In contrast, the M2 phenotype represents anti-inflammatory ("alternatively" activated) macrophages. This phenotype can be subdivided into several subtypes with diverse functions. This includes, for instance, immunosuppressive tumor-associated macrophages (TAM), or the pro-resolving macrophages participating in the resolution phase of inflammation. However, the transcriptomic signature of mouse pro-resolving macrophages differs from those of "M2-like" macrophages (36). This attests that pro-resolving macrophages of the resolution phase belong to a specific macrophage subtype, consistent with the "spectrum" model.

As mentioned above, macrophages are plastic cells highly sensitive to their microenvironment. Cells dying by apoptosis create an anti-inflammatory microenvironment that may affect neighboring macrophages. Thus, soluble factors released by apoptotic cells may stimulate macrophage reprogramming (Figure 1A). These factors released by apoptotic cells have been proposed to be used therapeutically to promote the resolution of inflammation (8, 37, 38). The administration of apoptotic cells themselves has been tested in experimental models of chronic inflammatory diseases (e.g., collagen-induced arthritis [CIA] (39), as well as in clinical settings (40, 41). We were the first in 2001 to propose the use of apoptotic cells as a cell-based therapy approach (42). However, this is out of the scope of this review (for recent reviews please refer to (21, 24, 43). Here, we want to focus on the contribution of macrophages to the resolution therapy.

Mechanisms leading to the control of macrophage reprogramming and those triggering the secretion of pro-resolving factors after efferocytosis are now beginning to be elucidated. Two main types of mechanisms have been identified so far. First, mechanisms delivered by apoptotic cells themselves (Figures 1A, B). This corresponds to soluble factors released by these dying cells (Figure 1A) and the direct interactions implicating cognate receptors expressed by apoptotic cells/bodies (including apoptotic cell-derived extracellular vesicles) and macrophages, respectively (Figure 1B). Second, the digestion of apoptotic cell-derived materials by efferocytic macrophages stimulates their reprogramming, and then the release of pro-resolving factors (Figure 1C). A synergy between these two mechanisms also exists (Figure 2A). A cooperation between apoptotic cells and macrophages before the engulfment of apoptotic cells may also generate pro-resolving factors (Figure 2B), such as adenosine. This nucleoside derives from adenosine monophosphate (AMP) released by apoptotic cells, which is then converted to adenosine by efferocytic macrophages (44). Production of SPM may also involve the cooperation of apoptotic cells and macrophages (6, 45). A last possibility to allow macrophages to generate pro-resolving mediators may result from the microenvironment in which the cells die (Figure 2C). Before discussing these mechanisms participating in macrophage reprogramming, we have to mention the three pioneer studies that initiated the elucidation of macrophage reprogramming and its role in the resolution of inflammation. The first study has proposed the critical role of apoptotic neutrophil elimination by macrophages to stop inflammation (13). The other two reported the synthesis by efferocytic myeloid cells of IL-10 (46) and TGF-β (19), two major anti-inflammatory cytokines associated with macrophage reprogramming. These cytokines are among the main factors studied that contribute to macrophage reprogramming.

2.1 Macrophage reprogramming induced by soluble factors released by apoptotic cells

Apoptotic cells create a local transient immunosuppressive microenvironment to prevent undesirable immune responses. This microenvironment does not affect only macrophages. Apoptotic cells may prevent the attraction of different immune cells by neutralizing inflammatory chemokines *via* the upregulation of CCR5 expression (47). In addition, apoptotic cells release soluble factors, including anti-inflammatory cytokines (e.g., TGF-β (48) or IL-10 (49), pro-resolving mediators (e.g., annexin-A1 (38) or thrombospondin-1 [Thbs1] (50)), as well as cellular metabolites (e.g., spermidine (37)). Some of these factors may induce macrophage reprogramming. For instance, together with M-CSF, IL-10 participates in the differentiation of human anti-inflammatory macrophages (named M2c according to the old nomenclature) (51). IL-10 upregulates the MerTK efferocytic receptor and the release of the bridging molecule, GAS6 (51), thereby promoting a higher capacity to clear apoptotic cells (52) (*i.e.*, "continual efferocytosis", see below). Apoptotic cells have been also reported to secrete "find-me" signals in order to attract professional phagocytes (e.g., monocyte-derived macrophages) to the site where the cells die. Among these "find-me" signals, some of them have immunomodulatory properties that may

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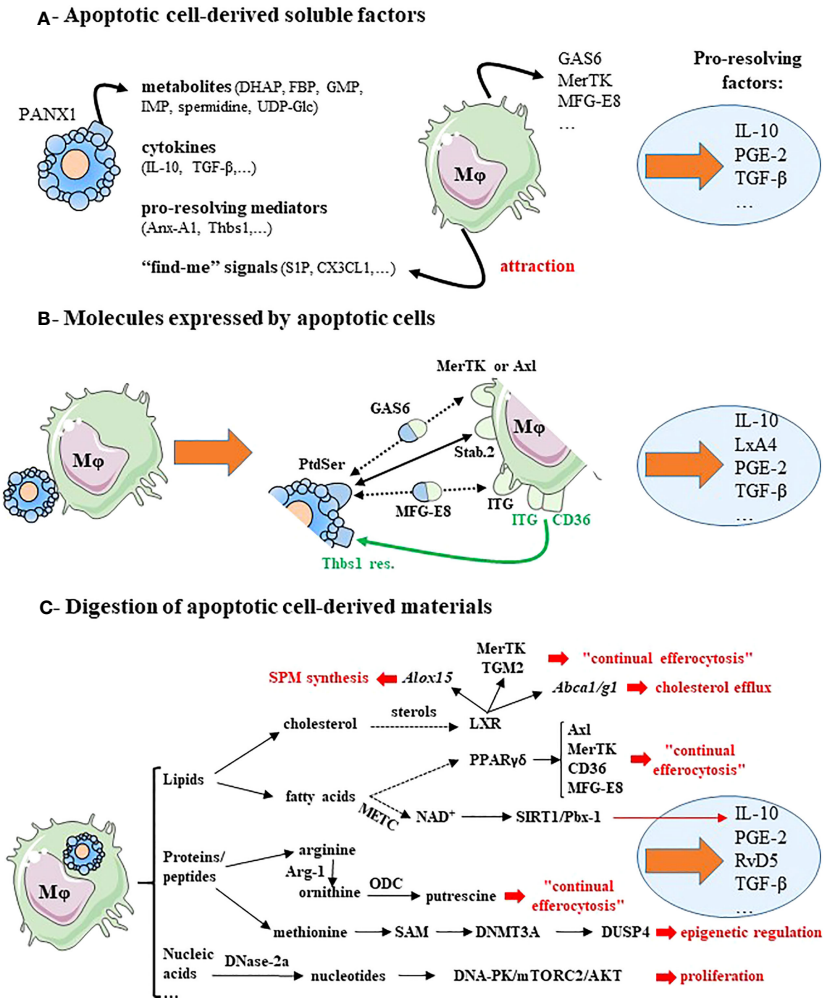

FIGURE 1
Macrophage reprogramming after efferocytosis may result from apoptotic cell-derived factors, surface molecules expressed by apoptotic cells or metabolites generated by apoptotic cell digestion. **(A)** Soluble factors released by apoptotic cells induce pro-resolving macrophage reprogramming and lead to pro-resolving factor secretion. Apoptotic cells release four kind of factors: cellular metabolites *via* the Pannexin-1 channels (PANX1), anti-inflammatory cytokines, pro-resolving mediators and "find-me" signals. These "find-me" signals affect tissue-resident macrophages locally, and diffuse to target monocyte-derived macrophages and attract them. These mediators stimulate the expression of efferocytic receptors (MerTK) and opsonins (e.g., GAS6 or MFG-E8). **(B)** Molecules expressed by cells becoming apoptotic promote macrophage reprogramming and induce the synthesis of pro-resolving factors. This is illustrated by the expression of the chief "eat-me" signal phosphatidylserine (PtdSer) that interacts directly with efferocytic receptors (e.g., Stabilin-2 [Stab.2]), or indirectly *via* bi-functional opsonins (e.g., GAS6 or MFG-E8) and with the efferocytic receptors MerTK or integrin receptors (ITG)(black lines). CD36 in association with integrin receptors (ITG, ITGB3 or ITGA5) recognizes thrombospondin-1 residues (Thbs1 res.) at the apoptotic cell surface (green lines). **(C)** Cellular metabolites resulting from apoptotic cell-derived materials stimulate macrophage reprogramming and the secretion of pro-resolving factors. In addition, these metabolites trigger several signaling pathways, implicated in efficient reprogramming including: macrophage proliferation, epigenetic regulation, continual efferocytosis, cholesterol efflux and specialized pro-resolving lipid mediator (SPM) synthesis (text in red font). Dotted arrows correspond to a suspected and indirect link. Pro-resolving factors are identified by light blue circles. Gene names are written in italics. For more details, please see the text. Abbreviations: ABC, ATP-binding cassette transporters; Alox15, arachidonate 15-lipoxygenase; Anx-A1, annexin-A1; Arg-1, arginase-1; CX3CL1, fractalkine; DHAP, dihydroxyacetone phosphate; DNA-PK, DNA-dependent protein kinase; DNase-2a, deoxyribonuclease-2a; DNMT3A, DNA methyltransferase-3A; DUSP4, dual-specific phosphatase 4; FBP, fructose-1,6-biphosphate; GAS6, growth arrest-specific protein 6; GMP, guanosine-5′-monophosphate; IMP, inosine-5′-monophosphate; ITG, integrin receptors;, LxA4, lipoxin-A4; LXR, liver X receptor; METC, mitochondrial electron transport chain; Mφ, macrophage; MFG-E8, milk fat globule-EGF factor 8; NAD^+, nicotinamide adenine dinucleotide coenzyme; mTORC2, mammalian Target Of Rapamycin complex-2; ODC, ornithine decarboxylase; PANX1, pannexin-1; PGE-2, prostaglandin-E2; PPAR, peroxisome proliferator-activated receptor; PtdSer, phosphatidylserine; RvD5, resolvin D5; S1P, sphingosine-1-phosphate; SAM, S-adenosylmethionine; SIRT1, sirtuin-1; Stab.2, stabilin-2; TGF-β, transforming growth factor-β; TGM2, transglutaminase-2; Thbs1, thrombospondin-1; Thbs1 res., thrombospondin-1 residues; UDG-Glc, uridine-diphosphate-glucose. This figure was depicted, in part, by using Servier Medical Art, https://smart.servier.com/.

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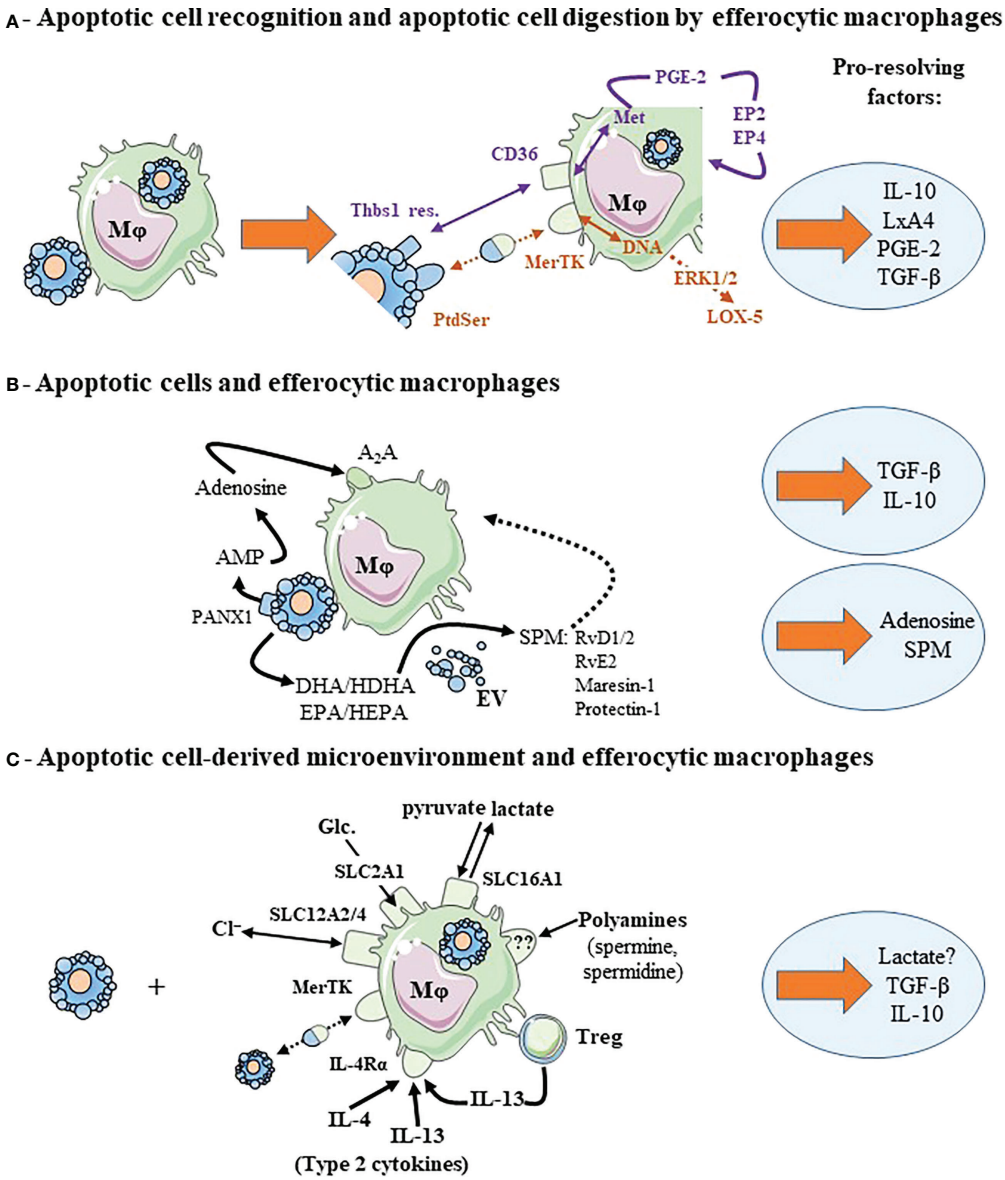

FIGURE 2
Cooperative mechanisms implicating efferocytic macrophages contributes to macrophage reprogramming and to the release of pro-resolving factors. **(A)** Recognition of apoptotic cell surface molecules by their cognate receptors and metabolites generated from digested apoptotic cells cooperate to induce macrophage reprogramming and pro-resolving factor release. Two examples are given corresponding to two different colors: DNA and recognition of PtdSer by MerTK in brown color, and methionine (Met) and recognition of thrombospondin-1 residues (Thbs1 res.) by CD36 in purple. We illustrated also the autocrine loop leading to TGF-β secretion implicating PGE-2 and its receptors, EP2 and EP4. **(B)** Efferocytic macrophages cooperates with apoptotic cells. Each cell contributes to macrophage reprogramming and the secretion of pro-resolving factors. This is the case of adenosine conversion by macrophages from AMP (adenosine mono-phosphate) released in the extracellular milieu by apoptotic cells through the Pannexin-1 (PANX1) channels. Apoptotic neutrophils or extracellular vesicles (EV) issued from these apoptotic neutrophils participate in the transcellular biosynthesis of specialized pro-resolving lipid mediators (SPM) by macrophages. Apoptotic neutrophils or EV provide SPM precursors (not necessarily DHA or EPA, but other intermediate precursors [HDHA and HEPA, respectively]) that are transformed in SPM by efferocytic macrophages. **(C)** Efferocytic macrophages may require signals from their microenvironment to acquire a pro-resolving profile and secrete pro-resolving factors. This microenvironment may correspond to ions (chloride, Cl^-), Glucose (Glc.) or their metabolites regulated through solute carrier transporter (SLC) exchange, polyamines, the presence of type 2 cytokines or regulatory T cells (Treg). Dotted arrows correspond to a suspected and indirect link. Pro-resolving factors are identified by light blue circles. For more details, please see the text. Other abbreviations: A2A, Adenosine 2A receptor; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; EP, prostaglandin-E2 receptor; ERK, extracellular signal-regulated kinase 1/2; HDHA, hydroxy-docosahexaenoic acid; HEPA, hydroxy-eicosapentaenoic acid; IL-4Rα, interleukin-4 receptor-alpha; LxA4, Lipoxin A4; Mφ, macrophage; PGE-2, prostaglandin-E2; RvD1/2, resolvin D1 and resolvin D2; RvE2, resolvin E2. This figure was depicted, in part, by using Servier Medical Art, https://smart.servier.com/.

stimulate macrophage reprogramming (53). For instance, the lipid sphingosine-1-phosphate induces M2 macrophage reprogramming with an increased production of IL-10 and PGE-2 (54). The "find-me" signal, fractalkine (also known as CX3CL1), enhances macrophage efferocytosis *via* the increased secretion of milk fat globule-EGF factor 8 (MFG-E8) (55), and also stimulates TGF-β production by macrophages (53). Thus, the microenvironment created by apoptotic cells may induce macrophage reprogramming and generate pro-resolving factors (Figure 1A).

2.2 Macrophage reprogramming induced by molecules expressed by apoptotic cells

After the emission of the "find-me" signals, apoptotic cells express "eat-me" signals that are recognized by receptors present on efferocytic cells. This promotes apoptotic cell engulfment (56). The interactions between "eat-me" signals expressed by apoptotic cells and their cognate receptors expressed by macrophages can stimulate macrophage reprogramming. Here, we will focus on some efferocytic receptors that deliver a reprogramming signal in macrophages. We will not be exhaustive in the description of the numerous interactions occurring between apoptotic cells and macrophages. For that, we recommend the following recent reviews (17, 56–58).

We will take the example of the main "eat-me" signal, phosphatidylserine (PtdSer) that is normally confined to the inner leaflet of the plasma membrane of viable cells. Apoptotic cells externalize PtdSer to the outer leaflet, and thus, express high levels of PtdSer at their cell surface. The pioneer works of Fadok and Henson identified the recognition of PtdSer by macrophages as critical for apoptotic cell removal (59), the production of TGF-β (19), and the resolution of inflammation (60). Masking this PtdSer present at the cell surface of apoptotic cells prevents both efferocytosis and its associated anti-inflammatory response (61). Several receptors recognizing PtdSer have been identified [for review (53, 56–58)]. Some interactions between "eat-me" signals and their cognate receptors require bi-functional soluble bridging molecules (named also opsonins). The Complement component C1q is an opsonin binding to different "eat-me" signals expressed by apoptotic cells, including PtdSer (56). This opsonin is recognized by several macrophage receptors, such as LRP1 (CD91), CR1 (CD35) or SCARF-1 (56, 62). The recognition of apoptotic cells *via* C1q induces anti-inflammatory macrophage reprogramming with the production of IL-10 (63). Axl and MerTK, two members of the TAM receptor family, recognize indirectly PtdSer *via* the opsonins, GAS6 or protein S (58). The stimulation of MerTK by apoptotic cells induces the translocation of lipoxygenase-5 (LOX-5) from the nucleus to the cytosol leading to the synthesis of the SPM, lipoxin-A4 (LxA4) derived from arachidonic acid (AA) (64). This connects apoptotic cell "eat-me" signal, macrophages and resolution mediators SPM. Furthermore, LxA4 increases TGF-β secretion by mouse macrophages (65), connecting SPM to anti-inflammatory cytokines. Another bridging molecule, called MFG-E8, allows the interactions between PtdSer and integrin receptors (*i.e.*, $\alpha_v\beta_3$ [ITGB3], also known as the vitronectin receptor (66) or $\alpha_v\beta_5$ [ITGA5]) expressed by macrophages (53). These integrin receptors coupled with CD36 interact with the Thbs1 residues expressed at the cell surface of apoptotic cells. The engagement of these integrin receptors by apoptotic cells induces the production of TGF-β by macrophages (53). Stabilin-2, another PtdSer receptor involved in efferocytosis, induces TGF-β secretion after apoptotic cell recognition (67). Moreover, CD300 family members representing other PtdSer receptors may stimulate IL-10 production by macrophages (53). Overall, surface molecules expressed by apoptotic cells –in association or not with opsonins– favor macrophage reprogramming with the secretion of anti-inflammatory cytokines. This step is critical as attested by systemic autoimmune diseases occurring in MerTK-deficient mice (68), or when PtdSer is masked (61).

2.3 Macrophage reprogramming induced by the digestion of engulfed apoptotic cells

The third step involved in the elimination of apoptotic cells –after the emission of "find-me" signals and the expression of "eat-me" signals– is the digestion or degradation of apoptotic cell derived-materials. The digestion of these apoptotic cell-derived materials (e.g., lipids, proteins/peptides, or nucleic acids) by macrophages after efferocytosis leads to a huge number of cellular components that should be transformed (*i.e.*, metabolized) and recycled by macrophages, or alternatively excreted to avoid their accumulation. This process of apoptotic cell digestion by macrophages is critical to prevent exacerbated autoimmune responses (69, 70) and to trigger the resolution of inflammation. This step is necessary for macrophages to acquire a pro-resolving and pro-repair profile (53, 71). Apoptotic cell-derived components generated by apoptotic cell degradation in phagolysosomes of macrophages may be implicated in macrophage reprogramming.

2.3.1 Lipid metabolism

The digestion of apoptotic cell-derived lipids by macrophages may lead to the accumulation of cellular components, including cholesterol and fatty acids. These apoptotic cell-derived lipids may affect macrophage functions (70–72), including both macrophage reprogramming and the capacity to continue to eliminate apoptotic cells over time. This process has been called by Ira Tabas "continual efferocytosis" (17, 73). Cholesterol represents one of the major apoptotic cell-

derived component. The lysosomal acid lipase is critical to hydrolyze cholesterol in the lysosomes after efferocytosis (70). This allows macrophages to produce anti-inflammatory (oxy) sterols that are required for optimal LXR activation. This LXR pathway stimulates then cholesterol efflux *via* the synthesis of ATP-binding cassette (ABC) transporters (70). Macrophages, like most of the cells of our body, lack the capacity to breakdown cholesterol. This efflux of cholesterol *via* ABC transporters (*i.e.*, ABCA1 and ABCG1) is thus critical for macrophage homeostasis. Apoptotic cells induce their own clearance *via* the LXR pathway that increases the expression of efferocytic receptor MerTK (72). Sterols derived from apoptotic cell-derived cholesterol may activate LXR in human efferocytic macrophages (74). In turn, LXR activation induces the upregulation of arachidonate 15-lipoxygenase (ALOX15). This enzyme participates in the resolution of inflammation by triggering the synthesis of resolvin D5 (RvD5) (74). The stimulation of the LXR/retinoic acid receptor-α (RAR-α) pathway enhances also the uptake of apoptotic cells through the efferocytic receptor, transglutaminase-2 (TGM2) (75). Cholesterol derivatives issued from digested apoptotic cells may also promote this TGM2 pathway, which may participate in "continual efferocytosis". Until now, cholesterol and its derivatives that accumulate after efferocytosis have not been metabolically traced as coming specifically from apoptotic cells.

An enrichment of long-chain fatty acids is also found in efferocytic macrophages using unbiased liquid chromatography-tandem mass spectrometry (71). Fatty acid breakdown from ingested apoptotic cells may be responsible for this enrichment. These fatty acids may stimulate mitochondrial respiration and may be involved in a non-canonical anti-inflammatory signaling pathway. This pathway requires an intact mitochondrial electron transport chain and involves the nicotinamide adenine dinucleotide NAD^+ coenzyme, the Sirtuin-1 signaling protein, and the transcription factor Pbx-1 that controls *Il-10* gene expression (71). Fatty acids generated from ingested apoptotic cells could be also potential activators of peroxisome proliferator-activated receptors (PPAR) (76). PPARγ and PPARδ have been shown to regulate macrophage reprogramming after efferocytosis (77–80). These nuclear receptors induce an increased expression of efferocytic receptors (e.g., Axl, MerTK or CD36), and the release of opsonins (e.g., MFG-E8) facilitating the uptake of apoptotic cells (77, 79–81). In addition, LXR and PPAR have been shown to antagonize the prototypical pro-inflammatory transcription factor, NF-κB (82, 83), which regulates the synthesis of pro-inflammatory cytokines, IL-6 or TNF. This participates in macrophage reprogramming by blocking pro-inflammatory cytokine production. While the LXR pathway is clearly anti-inflammatory in mouse macrophages, this pathway may be also pro-inflammatory in human macrophages (23). Nevertheless, lipid metabolism resulting from apoptotic cell degradation may participate in macrophage reprogramming after efferocytosis and the production of pro-resolving factors (Figure 1C).

2.3.2 Amino acid metabolism

Amino-acid levels increase after efferocytosis as a consequence of apoptotic cell-derived protein/peptide degradation. This concerns, in particular arginine, ornithine, lysine and methionine, while no increase of alanine and glycine is detected (73, 84). Ornithine may result from apoptotic cell-derived arginine metabolized by arginase-1 (73). An increased arginase-1 activity in mouse efferocytic macrophages has been confirmed in another study (84). Then, ornithine may be transformed *via* ornithine decarboxylase (ODC) into putrescine. An increase of ODC activity after efferocytosis is detected (73). The arginase-1/ODC/putrescine pathway is implicated in "continual efferocytosis" in mouse macrophages (73). In contrast, the next metabolites after putrescine in the polyamine pathway (*i.e.*, spermidine and spermine, respectively) are not required for "continual efferocytosis" (73). The situation is a little bit more complex for human macrophages. Indeed, in contrast to data obtained in mouse pro-resolving macrophages, arginase-1 is not a marker of human pro-resolving macrophages (85). However, apoptotic-cell derived ornithine and its metabolite putrescine both contribute also to "continual efferocytosis" in human macrophages (73).

Another amino acid, methionine, is generated by the phagosomal degradation of apoptotic cell-derived proteins or peptides. Methionine is then converted into S-adenosylmethionine that is used by a DNA methyltransferase (DNMT) called DNMT3A. This enzyme transfers methyl groups to regulatory DNA regions leading to the suppression of gene transcription. Thus, dual-specific phosphatase 4 is repressed by DNMT3A after efferocytosis. This pathway is involved in macrophage reprogramming after efferocytosis by inducing the production of PGE2 and TGF-β (12). This work identifies an epigenetic regulation mechanism by which apoptotic cell-derived materials give macrophages a pro-resolving phenotype. This epigenetic regulation may induce prolonged anti-inflammatory macrophage reprogramming.

2.3.3 Nucleic acid degradation

Digestion of apoptotic cell-derived nucleic acids is critical to avoid exacerbated autoimmune responses (69). The levels of nucleotides resulting from nucleic acid degradation increase in efferocytic macrophages (84). This is the case of cytosine for instance (84). Apoptotic cell-derived nucleotides after the degradation of DNA by cellular deoxyribonuclease-2a activate the DNA-dependent protein kinase/mTORC2/phospho-AKT proliferation pathway (86). Thus, macrophages proliferate in response to efferocytosis. Efferocytosis-induced proliferation is required for macrophages to acquire their pro-resolving functions. Macrophages undergoing efferocytosis-induced proliferation continue to eliminate apoptotic cells and produce the two key anti-inflammatory cytokines associated with efferocytosis, TGF-β and IL-10 (86).

Overall, controlled degradation of apoptotic cell materials is required to drive the acquisition of pro-resolving properties and to prevent chronic inflammation. Metabolites generated from ingested apoptotic cells may trigger signaling pathways leading to pro-resolving factors (Figure 1C).

2.4 Macrophage reprogramming induced by cooperative mechanisms

In the next three paragraphs, we will discuss the cooperation of different mechanisms leading to the production of pro-resolving factors. The first associates mechanisms already described (2.3), while the other two cooperative mechanisms require a partnership between cells (*i.e.*, apoptotic cells and macrophages), or with their microenvironment (e.g., cellular metabolites, specific cytokines or immune cells). Understanding these cooperative mechanisms is highly pertinent for the discussion of the new pro-resolving drug candidate SuperMApo (section 3).

Macrophage reprogramming can be stimulated by the synergistic action of apoptotic cell recognition by macrophage efferocytic receptors and the degradation of apoptotic cell-derived components (Figure 2A). Indeed, the combined action of apoptotic cell recognition by macrophage receptor CD36 and the generation of methionine by phagolysosomal degradation of apoptotic cells are necessary for PGE2 and TGF-β production (12). TGF-β production results from an autocrine loop implicating PGE2 and its two receptors prostaglandin-E2 receptors 2 and 4 (EP2 and EP4) present on macrophage surface (12). In the same way, the simultaneous recognition of apoptotic cells by MerTK expressed by macrophages and the degradation of apoptotic cell-derived DNA in these cells are also needed for both macrophage proliferation in response to efferocytosis and anti-inflammatory macrophage reprogramming (86). This cooperation allows efferocytic macrophages to maintain a prolonged extracellular signal-regulated kinase 1/2 (ERK1/2) activation (86), necessary for macrophage proliferation (86), PGE-2 and TGF-β production (12), as well as an accumulation of LOX-5 in the cytosol (64). As a reminder, LOX-5 may act with ALOX15 to metabolize AA into LxA4. Altogether, multiple signals delivered by apoptotic cells themselves or their byproducts are involved in the reprogramming of macrophages into pro-resolving macrophages.

2.4.1 Cooperation of apoptotic cells and efferocytic macrophages

As discussed, apoptotic cells and macrophages may cooperate before the internalization of apoptotic cells to generate adenosine (Figure 2B). In this case, adenosine results from AMP released by apoptotic cells that is metabolized into adenosine by efferocytic macrophages (44). Thus, apoptotic cells and efferocytic macrophages act in a synergistic manner to produce adenosine, a well-known mediator of resolution (5, 87). Another situation of cooperation between macrophages and apoptotic cells leads to the production of SPM (Figure 2B) by a mechanism called trans-cellular biosynthesis (88). Indeed, the interactions of apoptotic neutrophils or apoptotic neutrophil-derived extracellular vesicles with macrophages increase the synthesis of different SPM, such as RvD1, RvD2, and RvE2 as well as maresin-1 and protectin-D1 (89, 90). In fact, apoptotic neutrophils or their extracellular vesicles provide an intermediate precursor –derived from the precursors (e.g., eicosapentaenoic acid [EPA] or docosahexaenoic acid [DHA])– that is converted into SPM by macrophages (90). These intermediate precursors are 15- and 12-hydroxy-EPA (HEPA) derived from EPA for E-series resolvins and 17- and 14-hydroxy-DHA (HDHA) derived from DHA for D-series resolvins, protectin-1 and maresin-1 (6, 7, 88). In addition, apoptotic neutrophil-derived extracellular vesicles may stimulate macrophage SPM biosynthesis *via* a specific signaling pathway involving G-protein coupled receptor(s) (GPCR) (90). These SPM promote macrophage pro-resolving functions, including increased efferocytosis, and TGF-β and IL-10 production *via* specific GPCR (please refer to (88).

2.4.2 Cooperation of apoptotic cell-derived microenvironment with efferocytic macrophages

Solute carrier (SLC) transporters have been shown to regulate efferocytosis by macrophages (91, 92) and by type 1 conventional dendritic cells (cDC1) (93). These SLC transporters participate in the exchange of a huge number of substrates (*i.e.*, ions, sugars, nucleotides, amino acids) across membranes, including plasma membranes, but also intracellular organelle membranes. They control extracellular and cytosolic concentrations of substrates that modulate cellular metabolism and signaling. The expression of 33 SLC was found to be modulated after efferocytosis as analyzed by RNA sequencing (91). Four SLC have been specifically studied in the regulation of macrophage efferocytosis (91, 92). SLC2A1 (also known as GLUT1) is a transporter facilitating glucose uptake from extracellular milieu. Glucose uptake *via* SLC2A1 regulates efferocytosis (91). SLC16A1 is a plasma membrane proton-linked monocarboxylate transporter of pyruvate and lactate. This plasma membrane transporter mediates lactate release after efferocytosis. This lactate release is critical for macrophages surrounding efferocyting cells to acquire an anti-inflammatory profile. This is attested by the upregulation of *Tgfb1* and *Il10* mRNA in "bystander" macrophages (91). SLC12A4 and SLC12A2 are involved in chloride efflux and influx, respectively. SLC12A2 dampens efferocytosis, while SLC12A4 promotes it. Most importantly, SLC12A2 regulates the anti-inflammatory response induced by efferocytosis (92). Thus, efferocytosis and its associated anti-inflammatory effect are regulated by extracellular metabolites/substrates through

SLC transporters implicated in carbohydrate metabolism, intracellular pH and chloride exchange.

Polyamine import may also participate in macrophage reprogramming during efferocytosis (84). Indeed, arginine-derived polyamines, namely spermidine and spermine, increase specifically in efferocytic macrophages. This polyamine increase may not result from the retention of apoptotic cell-derived metabolites after their digestion, nor from the *de novo* biosynthesis of polyamines from arginine triggered by efferocytosis. In contrast, this accumulation of polyamines may arise from the import of polyamines present in the microenvironment. A Rac1- and actin-dependent endocytic process could be responsible for this import. The blockade of this endocytic import reduces polyamine accumulation, and prevents concomitantly macrophage reprogramming (*i.e.*, the inhibition of IL-1β and IL-6) induced by efferocytosis (84). The origin of spermine and spermidine present in the microenvironment of efferocytic macrophages remains to be determined. However, spermidine has been shown to be released by apoptotic cells themselves through pannexin-1 channels (37). Overall, in addition to apoptotic cells and macrophages, the microenvironment in which these cells are present may modulate macrophage reprogramming and the subsequent release of pro-resolving factors.

One has to specify that opposite results have been reported concerning ODC activity after efferocytosis [*i.e.*, reduced (84) *versus* increased activity (73)]. This may explain the need of polyamine import in the setting of reduced ODC activity (84), while increased ODC activity after efferocytosis may be able to furnish apoptotic cell-derived polyamines (73). This discrepancy could be explained by the type of macrophages used in the two studies, *i.e.*, "alternatively activated" M2 macrophages (73) *versus* M1 macrophages (84). Thus, the cytokine microenvironment (type 2 IL-4/IL-13 cytokines or type 1 IFN-γ cytokine) may influence macrophage metabolism and reprogramming after efferocytosis. The engagement of the IL-4 receptor-α (IL-4Rα) by type 2 cytokines (IL-4 or IL-13) together with the recognition of apoptotic cells by MerTK and Axl has been shown not only to cooperate to induce tissue repair, but also to increase *Alox15* transcript expression by mouse pro-resolving macrophages (94). The *Alox15* gene is the murine gene coding for 12/15-Lipoxygenase (12/15-LOX) (95). Its human ortholog is 15-LOX encoded by the *ALOX15* gene (95). These enzymes –human 15-LOX and mouse 12-15/LOX– mediate the oxidation of unsaturated fatty acids. Depending on its substrate (e.g., AA, or DHA), they generate different SPM, such as D-series resolvins, protectins, or lipoxins (95). Whereas mouse and human macrophages may respond differently to IL-4 (96), an increase of *ALOX15* expression in human macrophages has been reported after efferocytosis in a type 2 cytokine microenvironment (74, 96). Thus, apoptotic cell recognition in a type 2 cytokine microenvironment promotes a pro-resolving and tissue repair profile (Figure 2C).

A last example of the modulation of macrophage reprogramming by the microenvironment is the activity of regulatory FoxP3$^+$ CD4$^+$ T cells (Treg) that promote macrophage efferocytosis *via* the type 2 cytokine IL-13 (97). Treg stimulate the IL-10 signaling cascade in macrophages (97). Thus, protagonists in addition to the two key players, apoptotic cells and macrophages, may participate in macrophage reprogramming and the secretion of pro-resolving factors (Figure 2C).

3 An example of pro-resolving factors released by macrophages after efferocytosis

Several criteria/functions have been proposed to define pro-resolving mediators. Five to eight criteria characterize these mediators, according to different authors (5, 10, 98). However, all these authors agree that not all these criteria are necessary to describe a pro-resolving mediator. The most frequent criterion is the inhibition of neutrophil trafficking, which stops to fuel the onset phase of inflammation. The other main functions of pro-resolving mediators are the following: the induction of neutrophil apoptosis, stimulation of macrophage efferocytosis (that could be assimilated as "continual efferocytosis"), macrophage reprogramming toward an anti-inflammatory or pro-resolving profile, inhibition of monocyte migration and stimulation of tissue repair (5, 10, 98). Pro-inflammatory cytokine scavenging can be considered as the last criterion (5). Additional factors released by efferocytic macrophages, such as anti-inflammatory cytokines (*i.e.*, IL-10 or TGF-β) are not pro-resolving mediators *per se*, but have been frequently reported in the different studies (Figure 1 and Figure 2). They exert a wider array of functions than pro-resolving mediators, and these functions are not always beneficial for our body, such as fibrosis for TGF-β (99) or excessive transient immunosuppression. Another feature shared by all the different drug candidates of resolution therapy is the need for their administration at the "right place and at the right time" (7). Moreover, some SPM are highly labile and they are quickly degraded. Under physiological conditions, pro-resolving factors are produced transiently during a given time until the end of inflammation.

In the last part of this manuscript, we will focus on a new drug candidate for resolution therapy; this consists of the secretome of macrophages having ingested and eliminated apoptotic cells (100). This drug candidate emerges directly from the critical role of pro-resolving efferocytic macrophages in the resolution of inflammation. Data obtained with other pro-resolving mediators, such as SPM could be briefly analyzed to determine the relevant experimental models and the future therapeutic indications (for details, please refer to a recent review (7)). In

their Table 2 (7), the authors summarize the effects of different SPM obtained in experimental models, as well as the dose used to achieve beneficial effects. The main indications are chronic diseases resulting from non-resolving inflammation, including asthma or CIA (a mouse model for RA). The authors also mention three ongoing clinical trials; two of which use pharmaceutical derivatives of RvE1 (*i.e.*, RX-10045 and RX-10001) and one study using a LxA4 analog, BLXA4-Me (7). The indications are the following: signs and symptoms of dry eye (NCT00799552, available on the clinicaltrial.gov website), gingivitis (NCT02342691), and a single and multiple ascending oral dose study in healthy volunteers (NCT00941018). In addition to this review (7), a recent study reports the use of another SPM maresin-1 in experimental autoimmune encephalomyelitis (EAE), a mouse model for MS (101). Besides these studies reporting the use of SPM in animal models of chronic diseases associated with non-resolving inflammation, SPM (7) –like other pro-resolving mediators (9)– have been tested in experimental peritonitis in order to assess their impact on neutrophils present in the peritoneal exudate and the kinetics of their elimination by macrophages. This allows researchers to evaluate different criteria of pro-resolving mediators, namely inhibition of neutrophil attraction, induction of neutrophil apoptosis and stimulation of macrophage efferocytosis. Two main peritonitis models have been used to validate the different mechanisms and signaling pathways involved in macrophage reprogramming after efferocytosis. This consists in zymosan A-induced (12, 36, 44, 64, 73, 84, 86, 97) and thioglycollate-induced (9, 84, 94) peritonitis. Indeed, after the initial infiltration of neutrophils, these cells become apoptotic and this recapitulates the different steps of efferocytosis. These models of peritonitis resolve themselves spontaneously in wild type mice. Mouse ligature-induced periodontitis is another model (102) used to identify and/or test pro-resolving mediators (9), since dysregulated inflammation is considered as a major initial pathophysiological mechanism in this disease (103). Altogether, the development of new drug candidates in the setting of resolution therapy requires the assessment of the effects of these candidates in experimental models of acute and chronic inflammation (e.g., peritonitis *versus* RA or asthma). Now, we will report our own experience with the drug candidate called SuperMApo®.

The SuperMApo drug candidate consists of the secretome of macrophages co-cultured with apoptotic cells at a ratio of 1:5 for 48 hours (100, 104, 105). In this setting, apoptotic cells are totally eliminated in 24 hours. SuperMApo has been generated using human and mouse cells, and tested in different xenogeneic and mouse models of acute and chronic inflammation (100, 104, 105). Mouse SuperMApo is generated using thioglycollate-elicited mouse macrophages co-cultured with mouse apoptotic thymocytes (rendered apoptotic by a 35 Gray-irradiation), whereas M2 (M-CSF-treated) monocyte-derived human macrophages cultured with human apoptotic lymphocytes allow us to generate human SuperMApo (100). Our first experiments showed that SuperMApo could be a pro-resolving complex biological drug candidate limiting inflammation in the acute inflammatory model of peritonitis (Table 1). The SuperMApo drug candidate demonstrates pro-resolving properties in the thioglycollate-induced peritonitis model (100). Moreover, SuperMApo enhances the efferocytic capacity of macrophages both *in vivo* and *in vitro* (100). Several groups concur that this represents one criterion for pro-resolving mediators (5, 10, 98). Furthermore, SuperMApo induces macrophage reprogramming (100), which is another criterion for pro-resolving mediators. Pro-resolving properties have also been identified in xenogeneic thioglycollate-induced peritonitis using human SuperMApo administrated in immunodeficient NOG mice reconstituted with both human peripheral blood mononuclear cells (PBMC) and polymorphonuclear neutrophils (PMN) (100). Finally, SuperMApo stimulates tissue repair in a mouse model of wound healing (104)(Table 1). Thus, SuperMApo exhibits at least three criteria of pro-resolving mediators and could be considered as a promising pro-resolving complex biological drug candidate.

Concerning experimental models of chronic diseases associated with non-resolving inflammation, SuperMApo has been tested in the mouse model of RA, CIA (100), as well as in different models of IBD, including dextran sodium sulfate (DSS)-induced and naive T cell transfer-induced colitis (104), as well as DSS-induced xenogeneic colitis (100). The capacity to reduce chronic inflammation has also been evaluated in the setting of cancer (105). SuperMApo has also been tested in xenogeneic graft-*versus*-host disease (GvHD) (100), and a biopsy forceps-wounded colonic mucosa model (104). The main results are summarized in Table 1 together with the conditions of administration (e.g., route and timing of administration, dose, etc.). A therapeutic effect (*i.e.*, the reduction of the clinical score in already established diseases) has been achieved in CIA, naive T cell-transfer-induced colitis and DSS-induced xenogeneic colitis. Thus, SuperMApo is able to control ongoing disease, clearly important for the clinical situation. Furthermore, based on data obtained in xenogeneic GvHD (Table 1), SuperMApo could be used to prevent acute GvHD, a major complication of allogeneic hematopoietic cell transplantation (106). Since the transplantation is always a scheduled procedure in patients, SuperMApo could be administered at the time of transplantation to reduce this GvHD and the associated morbidity and mortality (106). Altogether, this demonstrates that SuperMApo is a potential new resolution therapy for chronic diseases associated with non-resolving inflammation, as well as acute GvHD.

The SuperMApo secretome contains large quantities of the anti-inflammatory cytokines TGF-β and IL-10, the IL-1 antagonist IL-1RA, as well as three chemokines CCL5, CXCL2 and CCL22 (100). TGF-β present in SuperMApo plays a crucial role, as demonstrated by blockade and depletion experiments (100, 104). However, other factors present in SuperMApo are

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TABLE 1 Consequences of SuperMApo treatment in different experimental models of acute and chronic inflammation.

Experimental model*	Conditions of administration (route, timing,…)	Main effects	Final results	Reference
Models of chronic inflammatory diseases resulting from non-resolving inflammation				
Collagen-induced arthritis (DBA/1 mice)	IV or IP – 10 injections (200 µL/mouse) each day for the first 5 injections, then, every 2 days or when SuperMApo is lyophilized and concentrated 5 times: 2 injections at two days apart – treatment starts at day 35 after CIA induction	- generation of collagen-specific Treg - pro-tolerogenic reprogramming of macrophages and pDC - mediated at least by TGF-β - no immunosuppressive effect (as attested by allogeneic skin graft rejection and survival after CLP)	- therapeutic effect on established arthritis up to 60 days following administration	(100)
DSS-induced colitis (C57Bl/6 mice)	IP – 2 injections (1 mL/mouse) the day of the first DSS cycle and 48 hr later		- improved endoscopic score and colon length - reduced clinical score	(104)
T cell transfer-induced colitis (RAG2$^{-/-}$C57Bl/6 mice)	IP – 2 injections (1 mL/mouse) at day 10 after T cell transfer and 48 hr later	- improved intestinal barrier integrity - increased cell proliferation within the intestinal crypts - increased activation of colonic fibroblasts - decreased expression of *Fn1* mRNA coding for extracellular matrix-associated fibronectin	- improved endoscopic and histological score, as well as colon length - reduced weight loss, and clinical score	(104)
DSS-induced xenogeneic colitis (human PBMC/NSG mice)	IP - 2 injections at days 14 and 16 (1 mL/mouse, 3 times concentrated human SuperMApo)		- improved survival and endoscopic score - reduced weight loss and clinical score	(100)
Cancer-induced chronic inflammation (EL4 mouse lymphoma line/C57Bl/6 mice)	IP – 2 injections (1 mL/mouse, 2 days apart) 7 days after EL4 injection	- increased macrophage mobilization in the tumor sites - reduced circulation of myeloid-derived suppressive cells - increased IFN-γ-specific anti-tumor response	- reduced cancer progression and dissemination	(105)
Models of acute inflammation				
Thioglycollate-induced peritonitis (C57Bl/6J mice)	IP – 1 injection at day 0	- enhanced efferocytic capacities of macrophages - anti-inflammatory macrophage reprogramming	- improved resolution	(100)
Thioglycollate-induced peritonitis (human PMBC and PMN/ NOG mice)	IP – 1 injection at day 0 (human SuperMApo)	- enhanced efferocytic capacities of macrophages - anti-inflammatory macrophage reprogramming	- improved resolution	(100)
Xenogeneic GvHD (human PBMC/NOG mice)	IP - 1 injection (1 mL/mouse, 5 times concentrated human SuperMApo) at day 0		- improved survival - reduced clinical score	(100)
Model of wound healing/tissue repair				
Biopsy forceps-wounded colonic mucosa model	IP – 2 injections (1 mL/mouse) the day of injury and 48 hr later	- increased proliferating (Ki67$^+$) intestinal cells - increased colonic fibroblast activation (α-SMA$^+$) with reduced expression of extracellular matrix associated gene *Fn1* - Limited destruction of intestinal barrier (Reg3γ, serum FITC-dextran)	- increased wound healing (assessed by video-endoscopy)	(104)

*mouse strain is provided for each experimental model. Abbreviations: α-SMA, α-smooth muscle actin; CLP, cecal ligation and puncture-induced sepsis; DSS, dextran sodium sulfate; IP, intraperitoneal; *Fn1*, the gene coding fibronectin; FITC-dextran, fluorescein-isothiocyanate-labeled beads; GvHD, graft-*versus*-host disease; IV, intravenous; NOG mice, *NOD.Cg-Prkdc^{scid}Il2rg^{tm1Sug}/ShiJic* mice; NSG mice, *NOD.Cg-Prkdc^{scid}Il2rg^{tm1Wjl}/SzJ* mice; PBMC, peripheral blood mononuclear cells; pDC, plasmacytoid dendritic cells; PMN, polymorphonuclear neutrophils; Reg3γ, regenerating islet-derived 3γ; Treg, regulatory FoxP3$^+$ CD4$^+$ T cells.

needed. The administration of the six factors mentioned above, as recombinant proteins, even used 3 times concentrated, does not recapitulate the therapeutic effect of SuperMApo in CIA (100). The presence of cofactors complexed with TGF-β in SuperMApo has been identified using biochips coated with anti-TGF-β monoclonal antibody, surface plasmon resonance experiments, and mass spectrometry analysis. Apoliprotein E, the complement component C1q, macrophage metalloelastase MMP12, Thbs1 and transthyretin are associated with TGF-β within SuperMApo. However, their administration together with recombinant TGF-β again has no therapeutic effect on CIA, in contrast to the administration of SuperMApo (100). In addition, TGF-β present in SuperMApo is critical to treat experimental colitis (104). Nevertheless, other growth factors present in this secretome of macrophages eliminating apoptotic cells participate in this effect. This consists of insulin-growth factor-1 (IGF-I) and vascular endothelial growth factor (VEGF) (104). Yet, TGF-β, IGF-I and VEGF participate in intestinal mucosal healing induced by SuperMApo; but they are not sufficient to resolve global intestinal inflammation and do not replace SuperMApo (104). Overall, SuperMApo contains multiple factors (approximately 500 factors), which act together to exert a therapeutic effect with TGF-β playing a central role. Some of these factors have been reported to be released by efferocytic macrophages and to exert anti-inflammatory functions (*i.e.*, TGF-β (12, 19, 53, 67, 86), IL-10 (46, 53, 54, 71, 86, 97) or IL-1RA (107)). Macrophages are able to produce C1q (108), and this complement component induces the anti-inflammatory reprogramming of macrophages (63). Among the three chemokines identified in high amounts in SuperMApo, CCL5 and CXCL2 may exert pro-inflammatory functions. However, previous transcriptomic analysis of different mouse macrophage subsets shows that pro-resolving macrophages are enriched in *Ccl5* compared with pro-inflammatory macrophages (36). CCL5 may participate in tissue regeneration by recruiting stroma cells *via* CCR1 (109). Moreover, CCL5 released by M2 macrophages may improve skin wound healing (110). In contrast, the increased production of CCL5 together with type I interferon after fungal infection may be responsible for impaired mucosal healing in Crohn's disease patients and in mice (111). CCL5/RANTES is chemotactic for type 1 (Th1) $CD4^+$ T cells, monocytes, dendritic cells, and NK cells *via* the expression of its receptors CCR1 and/or CCR5 (112–115) and thus may mediate pro-inflammatory effects. In the same way, CXCL2 induces the recruitment of neutrophils (116), which may fuel the inflammatory response. Transthyretin, an amyloidogenic protein found complexed with TGF-β in SuperMApo, may also promote a pro-inflammatory response. Indeed, aggregated transthyretin stimulates the progression of osteoarthritis in mouse models. However, this requires the intra-articular injection of aggregated purified transthyretin. The same injection of non-aggregated transthyretin does not induced synovitis (117). Alternatively, the factors identified in SuperMApo may represent pro-resolving mediators, such as Thbs1 (118). Until now, efforts to use the association of multiple recombinant proteins found in SuperMApo to treat chronic inflammatory diseases have been a failure. At this stage, the complete secretome in its native form should be used to resolve uncontrolled inflammation.

Concerning the mechanisms of action, the interactions of SuperMApo with innate immune cells, adaptive immune cells and cells involved in tissue repair have been studied. This has been done *in vivo* in experimental models, but also in relevant *in vitro* assays. In peritonitis models, SuperMApo stimulates the recruitment of both human and mouse neutrophils (100). This could be related to the high amount of CXCL2 found in SuperMApo (100), since CXCL2/MIP-2 has been shown to induce mouse neutrophil recruitment in the peritoneum (116). Thus, SuperMApo exerts a pro-inflammatory function by attracting neutrophils, potentially *via* CXCL2. However, the promoting effect of SuperMApo on macrophage efferocytosis may allow these cells to rapidly eliminate apoptotic neutrophils and to accelerate the resolution phase of inflammation, as observed in the peritonitis models (100). In addition, SuperMApo administration promotes macrophage reprogramming in the CIA model (100). This means that factors released by efferocytic macrophages (*i.e.*, SuperMApo) are able to confer a pro-resolving phenotype to activated pro-inflammatory macrophages. This effect is dependent on TGF-β present in SuperMApo (100). However, as mentioned above, other factors may participate in this effect. One may hypothesize that lactate released by efferocytic macrophages may be one of these factors, since this metabolite has been reported to transfer macrophage reprogramming to "bystander" non-efferocytic macrophages (91). This mechanism has been proposed to explain the reprogramming of TAM (119). SuperMApo favors plasmacytoid dendritic cell (pDC) reprogramming in the CIA model, and these cells are involved in collagen-specific Treg induction observed after the administration of SuperMApo. This effect is also dependent on TGF-β present in SuperMApo (100). This is not surprising, since we previously reported in a bone marrow transplantation model that TGF-β released by efferocytic macrophages stimulates pDC to generate Treg polarization (120). This increase of Treg observed after the administration of SuperMApo could be also due to the presence of CCL22 in this secretome. Indeed, splenic marginal zone macrophages eliminating apoptotic cells secrete high amounts of CCL22, which, in turn, induces the recruitment of $CCR4^+$ Treg in the spleen (*i.e.*, the site of apoptotic cell removal) (121). After the administration of SuperMApo in the CIA model, both macrophages and pDC are implicated in the generation of autoantigen (collagen)-specific Treg and this requires TGF-β (100) (Table 1).

The administration of SuperMApo targets also non-immune cells involved in tissue repair. This was shown in the three different models of intestinal inflammation used whatever the

initial injury (mechanical [biopsy forceps], T cell-mediated or chemical [DSS]) (104) (Table 1). SuperMApo stimulates wound healing *via* the pro-healing properties of intestinal epithelial cells (IEC) and fibroblasts. *In vitro*, SuperMApo increases the proliferative and migratory properties of an IEC line and enhances its wound closure properties. TGF-β and IGF-I present in SuperMApo have been shown to participate in the SuperMApo-induced IEC proliferation. These two growth factors and VEGF are involved in the *in vitro* effect of SuperMApo on IEC migration (104). Moreover, SuperMApo allows IEC to acquire efferocytic properties (104). Boosting apoptotic cell clearance of colonic epithelial cells has been previously shown to dampen intestinal inflammation (122). However, it remains to be determined whether the increased efferocytic properties conferred by SuperMApo to a non-professional phagocyte, here IEC, may result from the presence in SuperMApo of opsonins (e.g., MFG-E8, GAS6 or C1q) or macrophage-derived extracellular vesicles –as previously reported for airway epithelial cells in the lungs (123).

The wound repair process occurs in three overlapping, but distinct phases. The proliferation and remodeling phases follow the inflammation one (124). TGF-β is a key activator of fibroblasts, which correspond to the central cellular effectors of fibrosis and tissue repair (99, 125). During the wound healing process, fibroblasts migrate in order to close the wound and they become activated. They differentiated into myofibroblasts with the acquisition of microfilament bundles constituted by α-smooth muscle actin (α-SMA) (126). SuperMApo stimulates the *in vitro* pro-healing properties of colonic fibroblasts, *i.e.*, their migration capacity in a wound-healing scratch assay and their contractibility (in a contraction assay using collagen culture gel). Furthermore, fibroblasts exposed to SuperMApo exhibit an activated phenotype (as attested by an increased expression of α-SMA) but demonstrate reduced pro-fibrotic functions (*i.e.*, a limited expression of extracellular matrix genes *Fn1*, *Col1a1*, and *Col3a1*). The *in vitro* migration properties of colonic fibroblasts in response to SuperMApo are reduced when one of the three growth factors identified in SuperMApo (*i.e.*, TGF-β, IGF-I, or VEGF) is depleted. These data reporting the role of growth factors in the effect of SuperMApo fit well with previous data showing that IGF-I and TGF-β induce fibroblast proliferation, and that TGF-β promotes fibroblast migration (99). Some of the *in vitro* data have been confirmed *in vivo* using the biopsy forceps-wounded colon model. This is the case of the proliferating properties of SuperMApo on intestinal cells, fibroblast activation and the reduced expression of the *Fn1* gene (104) (Table 1). Overall, SuperMApo exhibits tissue repair properties by targeting IEC and fibroblasts.

Resolution therapy has been proposed to be used in cancer (127), which belongs also to chronic inflammatory diseases associated with non-resolving inflammation (1). We observed that SuperMApo reduces tumor cell dissemination to the blood and mesenteric lymph nodes (105). Moreover, SuperMApo increases specific anti-tumor T cell responses. This increase of specific anti-tumor IFN-γ responses induced by the administration of SuperMApo was found correlated to the induction of macrophages highly expressing MHC class II molecules (105). The transcriptomic analysis of different macrophage subsets identified that mouse pro-resolving macrophages are enriched for genes coding for antigen processing and presentation (MHC class II genes [*H2-Aa*]) in comparison with naive macrophages (36). Thus, the preoperative administration of SuperMApo could be tested in cancers with an important inflammatory component. However, the mechanisms of action used by SuperMApo in this setting should be further deciphered.

Data obtained in experimental models are sometimes difficult to transpose to clinical settings. One explanation could be the difference between species concerning certain immune cell subsets. This has been reported concerning efferocytic macrophages. This concerns in particular the macrophage response to IL-4 (96), arginine metabolism (85) and the LXR signaling pathways that differ between human and mouse macrophages (23). Several studies considering anti-inflammatory macrophage reprogramming and the resulting pro-resolving factors compared mouse and human macrophages (12, 84, 86). Identical mechanisms were found, except for arginase-1 (73). Similar pro-resolving mechanisms are also reported in independent studies, such as the induction of *ALOX15* or of its murine ortholog *Alox15* by the simultaneous recognition of apoptotic cells in a type 2 cytokine microenvironment (*i.e.*, IL-4 or IL-13) (74, 94). We tested human SuperMApo in xenogeneic models and encouraging results were obtained (100) (Table 1). Based on SuperMApo content, potential adverse effects may occur after its administration. These may include excessive immune suppression or a pro-inflammatory effect related to factors present in SuperMApo. Despite the presence of high levels of TGF-β and IL-10, no immunosuppressive effect has been observed after the administration of SuperMApo in the CIA model. SuperMApo-treated mice are still able to reject a skin allograft and resist to sepsis-induced mortality in the same way as untreated mice (100). SuperMApo contains factors that may exert both pro-inflammatory and pro-resolving functions. This is the case of chemokines, CCL5 and CXCL2 or other factors such as transthyretin. To date, we did not observe any pro-inflammatory effects in the different experimental models used until now (100, 104). However, we should continue to carefully monitor for any potential pro-inflammatory consequences. An argument in favor of the initiation of clinical trials is data obtained with human fibroblasts isolated from patient colon biopsies. Inflamed lesion-derived human fibroblasts demonstrate *in vitro* proliferation and enhanced wound closure capacities in response to SuperMApo (104). Altogether, this supports the use of SuperMApo in clinical settings (e.g. RA and IBD). We propose to infuse SuperMApo

intravenously. To date, the precise infusion regimen in patients remains to be determined.

4 Conclusion

The resolution of inflammation is currently identified as an active process resulting from the effect of pro-resolving factors. These factors, including pro-resolving mediators, can be used therapeutically (3–5, 7) to treat chronic inflammatory diseases resulting from non-resolving inflammation (1). New treatments are also required in chronic diseases for which several therapeutic options exist, such as RA. While the management of RA has dramatically changed in the last 20 years with the use of TNF inhibitors, a substantial proportion of patients treated with these anti-TNF therapies still exhibits an inadequate response and does not achieve remission. Moreover, some patients develop undesirable side effects, such as infections. Thus, new therapies are still needed in RA (2). Our encouraging results obtained with SuperMApo in CIA support its use in RA. One may also extend the indications of resolution therapy to acute inflammatory diseases with non-resolving inflammation, such as the severe form of severe acute respiratory syndrome coronavirus (SARS-CoV2) infection (128). Indeed, the blockade of anti-inflammatory macrophage reprogramming by SARS-CoV2-infected apoptotic cells has been recently reported (128). The SARS-CoV2 hyper-inflammatory syndrome (129) could result from the absence of macrophage reprogramming after efferocytosis of SARS-CoV2-infected dying cells. Severe SARS-CoV2 infections may therefore be considered as another disease resulting from non-resolving inflammation (1), and could be treated with resolution therapy (as recently proposed (130)). SuperMApo constitutes a new pro-resolving complex biological drug candidate in the therapeutic arsenal of resolution therapy.

As discussed (section 3), resolution therapy has been proposed to be used in cancer (127). Indeed, aspirin has been shown to trigger the production of different SPM (e.g. RvD1 or LxA4), which stimulate cancer resolution by targeting macrophage subsets (131). The preoperative, but not postoperative, administration of a NSAID (*i.e.*, ketorolac) alone or associated with resolvins has been shown to eliminate micrometastases in different tumor-resection models (132). Furthermore, the preoperative stimulation of resolution using SPM, and more particularly D-series resolvins (RvD2, RvD3, and RvD4), inhibits metastases and promotes T cell responses (132). A recent review discusses the role of SPM to reeducate TAM in order to fight cancer (127). SuperMApo could be an additional therapy to limit cancer-induced chronic inflammation.

From a mechanistic point of view, the results currently obtained with SuperMApo in the different experimental models suggest that the effect of SuperMApo is disease-specific, targeting different pathogenic cell subsets. SuperMApo particularly affects innate and T cell responses in CIA, while intestinal-resident cells involved in tissue repair are the main target of SuperMApo in colitis (Table 1). This may reflect the different pathophysiological mechanisms of these two diseases and their different locations (*i.e.*, the joint *versus* the colon). Concerning the pro-healing properties of SuperMApo, these different mechanisms may be linked to fibroblast heterogeneity between organs (125, 133, 134). The evaluation of the administration of SuperMApo in experimental models of chronic diseases affecting other organs, for instance, EAE, could allow us to investigate these issues. Deciphering immune mechanisms resulting from the secretome of efferocytic macrophages may shed light on the role of efferocytic macrophages, their interactions with partner cells (e.g., neutrophils, fibroblasts or Treg) in chronic inflammation. SuperMApo may also enable to identify new pro-resolving factors.

An important and necessary future step will be to improve the characterization of SuperMApo. While TGF-β seems to be the key player in both CIA and colitis, its precise mechanism remains to be determined, in particular its association with other factors that bind to several proteins (e.g., the opsonins C1q or Thbs1). TGF-β is known to participle in the resolution of inflammation by exerting immune regulatory functions, which are critical for the return to homeostasis. However, it is not clear whether it acts directly by inducing leukocyte apoptosis and by decreasing the resolution index. This cytokine plays a main homeostatic role in the control of wound healing and tissue repair (135). Macrophages are the major source of TGF-β, which is secreted in its latent form. After secretion, latent TGF-β binds to collagens or proteins with collagen-rich regions (99), such as the Complement component C1q (62). TGF-β is activated by several mechanisms and factors. Thbs1 and MMP, both present in SuperMApo, can activate latent TGF-β (99, 126). Further experiments are required to decipher the precise mechanism(s) occurring in the secretome of efferocytic macrophages (*i.e.*, SuperMApo). Whereas TGF-β is profibrotic (99, 126), SuperMApo contains MMP which may participate in the destruction of extracellular matrix (124) and reduce the excessive deposition of extracellular matrix proteins. Whether SuperMApo contains cellular metabolites (e.g., lactate), SPM (or SPM intermediate precursors such as HDHA or HEPA), or extracellular vesicles remains to be determined. Lactate may participate in latent TGF-β activation (99).

To conclude, patients suffering from diseases with non-resolving inflammation have unmet medical needs. We propose that resolution therapy can help address this issue. This therapy will benefit from the significant advances performed in the understanding of efferocytosis and macrophage reprogramming. A recent research topic dealing with molecular and cellular effectors in the resolution of inflammation (136) could also facilitate the

development of resolution therapy. Furthermore, the analysis of the interactions between macrophages and fibroblasts in the setting of fibrosis could also favor the development of this therapy. SuperMApo could be a way to study these interactions and to better understand the role of efferocytosis in the resolution of inflammation.

Author contributions

All the authors performed the bibliographic search and participated in the draft of this review. PS wrote the first version of the manuscript, made the table and the figures. All authors contributed to the article and approved the submitted version.

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Conflict of interest

FB and SP are employed by MED'INN'PHARMA, which develops a pro-resolving drug candidate called SuperMapo®. SP, FB and PS are shareholder of MED'INN'PHARMA.

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

Publisher's note

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Role of FK506-sensitive signals in asthmatic lung inflammation

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Asthma is airway inflammatory diseases caused by the activation of group 2 innate lymphoid cells (ILC2s) and type 2 helper T (T_H2) cells. Cysteine proteases allergen cause tissue damage to airway epithelial cells and activate ILC2-mediated type 2 airway inflammation. FK506 is an immunosuppressive agent against calcium-dependent NFAT activation that is also effective against asthmatic inflammation. However, the effects of FK506 on cysteine protease allergen-mediated airway inflammation remain unclear. In this study, we investigated the suppressive effects of FK506 on airway inflammation. FK506 had a partial inhibitory effect on ILC2-dependent eosinophil inflammation and a robust inhibitory effect on T cell-dependent eosinophil inflammation in a cysteine protease-induced mouse asthma model. The infiltration of T1/ST2$^+$ CD4 T cells in the lungs contributed to the persistence of eosinophil infiltration in the airway; FK506 completely inhibited the infiltration of T1/ST2$^+$ CD4 T cells. In the initial phase, FK506 treatment targeted lung ILC2 activation induced by leukotriene B_4 (LTB_4)-mediated calcium signaling, but not IL-33 signaling. FK506 also inhibited the IL-13-dependent accumulation of T1/ST2$^+$ CD4 T cells in the lungs of the later responses. These results indicated that FK506 potently suppressed airway inflammation by targeting ILC2 activation and T1/ST2$^+$ CD4 T cell accumulation.

KEYWORDS

asthma, innate lymphocyte cells (ILCs), Th2 airway inflammation, cytokines, IL-13

Introduction

Asthma is a lifelong disorder usually driven by type 2 immune-inflammatory pathogenic mechanisms. The inflammatory responses observed in asthma are complex, and airway epithelial cells (AECs) are critical for the initial development of local inflammation (1). Several allergens, including house dust mites, food, and fungi,

possess group 1 cysteine protease activity, which increases the permeability of local epithelial cells. Tissue damage caused by cysteine proteases allergen allows the release of epithelial-derived cytokines, interleukin (IL)-33, IL-25, and thymic stromal lymphopoietin (TSLP), which activate group 2 innate lymphocytes (2). Damaged AECs release IL-33 and TSLP, basophils produce IL-4, and T_H2 cells produce type 2 cytokines, including IL-4, IL-5, and IL-13, which mainly contribute to allergic airway inflammation, subsequently leading to allergen-specific IgE-mediated mast cell degranulation and eosinophil recruitment (3–5). ILC2s secrete relatively high amounts of IL-5 and IL-13 (6–8). These ILC2-derived type 2 cytokines also play critical roles in hyperplasia, mucin formation in bronchial epithelial cells, and eosinophil accumulation (9, 10). ILC2-derived type 2 cytokines could also contribute to the accumulation of T_H2 cells in the local inflammation site (10–12). Recently, inflammatory mediators synthesized from arachnoid acids, such as prostaglandin D_2 (PGD_2) and leukotriene D_4 (LTD_4), have been reported to activate ILC2s (13, 14). Therefore, cysteine proteases allergen promote several aspects of asthmatic airway inflammation by activating innate immune responses.

Several treatments are generally used to control and prevent asthma attacks. Systemic treatment with steroids, such as corticosteroids, is ineffective in some patients with severe asthma (15). Moreover, the long-term use of steroids induces side effects (16). Leukotriene modifiers are a promising treatment to control asthma since they block the actions of leukotrienes, which tighten the airway muscles (17). An immunosuppressive agent, FK506, blocks calcium-dependent nuclear factor of activated T cells (NFAT) activation, and T-cell activation (18–20) is another promising treatment for asthma by targeting type 2 cytokine release from T_H2 cells (21, 22). In a mouse model of *Aspergillus*-induced asthma, FK506 targets chronic asthmatic inflammation, improving eosinophil infiltration (23). However, the effects of FK506 on ILC2 activation remain controversial. IL-33-dependent ILC2 activation is expected to be resistant to FK506, whereas lipid mediator-mediated activation *via* LTB_4, LTD_4, and LTE_4 is expected to be susceptible (24). Therefore, the precise effect of FK506 on the cysteine protease allergen-mediated airway inflammation remains unclear. In particular, it is unclear whether innate or adaptive immune responses are the primary targets of FK506 treatment.

In this study, we investigated the mechanism underlying the inhibitory effect of FK506 on cysteine protease allergen-induced airway inflammation in mice. We found that FK506 effectively inhibited ILC2 activation and $ST2^+$ CD4 T cells accumulation in the lung. ILC2s are the initial target of FK506, inhibiting their functions *via* calcium-dependent activation, including LTB_4-induced activation. These findings indicate that ILC2 and CD4 T cells are potential targets of FK506 and shed light on the mechanisms that inhibit airway inflammation induced by protease allergens.

Results

FK506 inhibits protease allergen-induced acute phase airway inflammation

We first investigated the effect of FK506 on T cell-independent airway inflammation caused by protease allergens in a papain-induced mouse model. Intranasal administration of papain for three days generated goblet cell hyperplasia and caused the infiltration of activated eosinophils in the lungs 24 h after the third injection. Papain treatment significantly increased eosinophil infiltration compared to PBS-treatment ($p<0.01$). In contrast, the intraperitoneal administration of FK506 partially reduced the number of infiltrating lung eosinophils ($p<0.01$) (Figure 1A) and significantly inhibited goblet cell generation and lymphocyte accumulation (Figure 1B). These inhibitory effects of FK506 were comparable to or slightly lower than those of dexamethasone, a corticosteroid (Figures 1A, B). These results indicate that FK506 inhibits protease-induced airway inflammation.

The acute airway response is T cell-independent and largely depends on innate immune cells, including basophils and ILC2s (25). Interestingly, the infiltration of $CD11c^+$ eosinophils in the lungs was sustained seven days after the initial papain treatment (Figure 2A). The persistence of eosinophil migration might influence the migration of CD4 T cells expressing the IL-33 receptor T1/ST2, which was not observed on day one (Figure 2A).

To further investigate the effect of FK506 on the day seven response, we analyzed the inhibitory effect of FK506 on the persistent activation of eosinophils on day 7. FK506 treatment attenuated the papain-induced infiltration of activated $CD11c^+$ eosinophils and accumulation of $T1/ST2^+$ CD4 T cells into the lungs (Figure 2A). Interestingly, the effect of dexamethasone on T cell migration was more subtle than that of FK506 (Figure 2A). Moreover, FK506 also inhibited papain-induced goblet cell hyperplasia and lymphocyte accumulation (Figure 2B). These results suggest that FK506 also inhibits the T cell-mediated persistence of eosinophil attraction. Similar $T1/ST2^+$ T cell migration with eosinophil attraction was recapitulated in mice nasally injected with IL-33 alone (Figure 2C), indicating that IL-33 receptor-expressing CD4 T cells, contributed to the persistence of eosinophil attraction.

Thus, we examined the role of $T1/ST2^+$ T cell cells using T cell-deficient mice ($Cd3^{-/-}$ mice). $Cd3^{-/-}$ mice showed papain-induced eosinophil infiltration on day one but did not show persistent infiltration of activated eosinophils on day seven

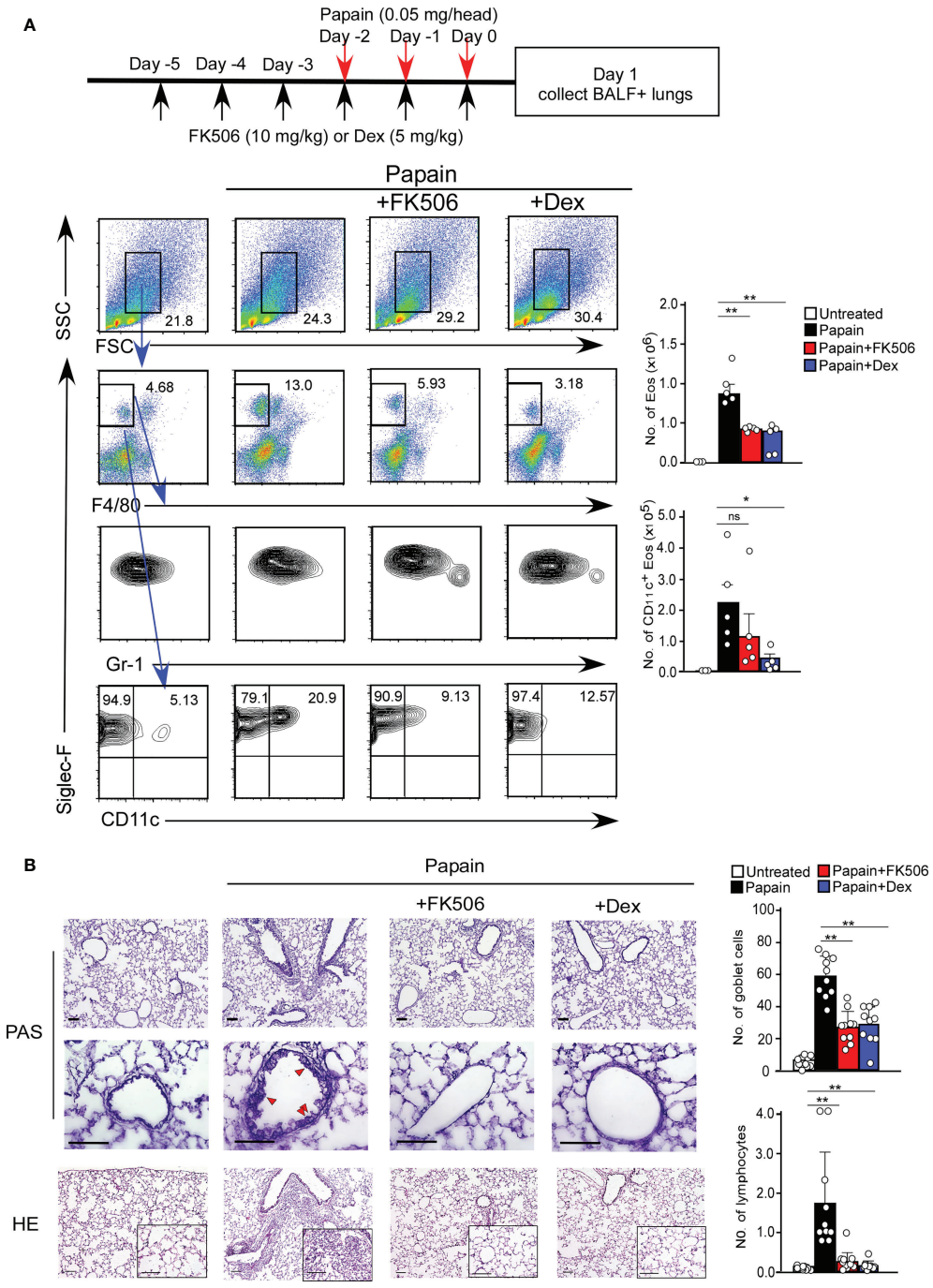

FIGURE 1
(A) The papain and FK506 or Dexamethasone (Dex) treatment protocol is presented by the schematic diagram (top). The mice were administrated with (black bars, n=5) or without (white bars, n=5) papain. Papain-administrated mice were further treated with FK506 (red bars, n=8) or Dex (blue bars, n=5). Lung cells were prepared from the lung homogenate on day one after the final papain injection (see Materials and Methods). Flow cytometry analysis assessed the percentage (left flow profiles) and cell numbers (right graphs) of Siglec-F+ and CD11c+ eosinophils (Eos). **(B)** PAS (top: low magnification; middle: high magnification) and HE (bottom) staining of lung sections from FK506- or Dex-treated mice. Red arrow heads indicate mucus glycans positive goblet cells. Goblet cells and lymphocytes were counted in 200 μm × 200 μm regions in each lung section (bar graphs, n=10). Bars represent the means; not significant (ns), *p−0.05, **p−0.01 using unpaired Mann-Whitney U-tests. All error bars represent SEM. Scale bars, 100 μm. FACS and section dates are representative of three experiments.

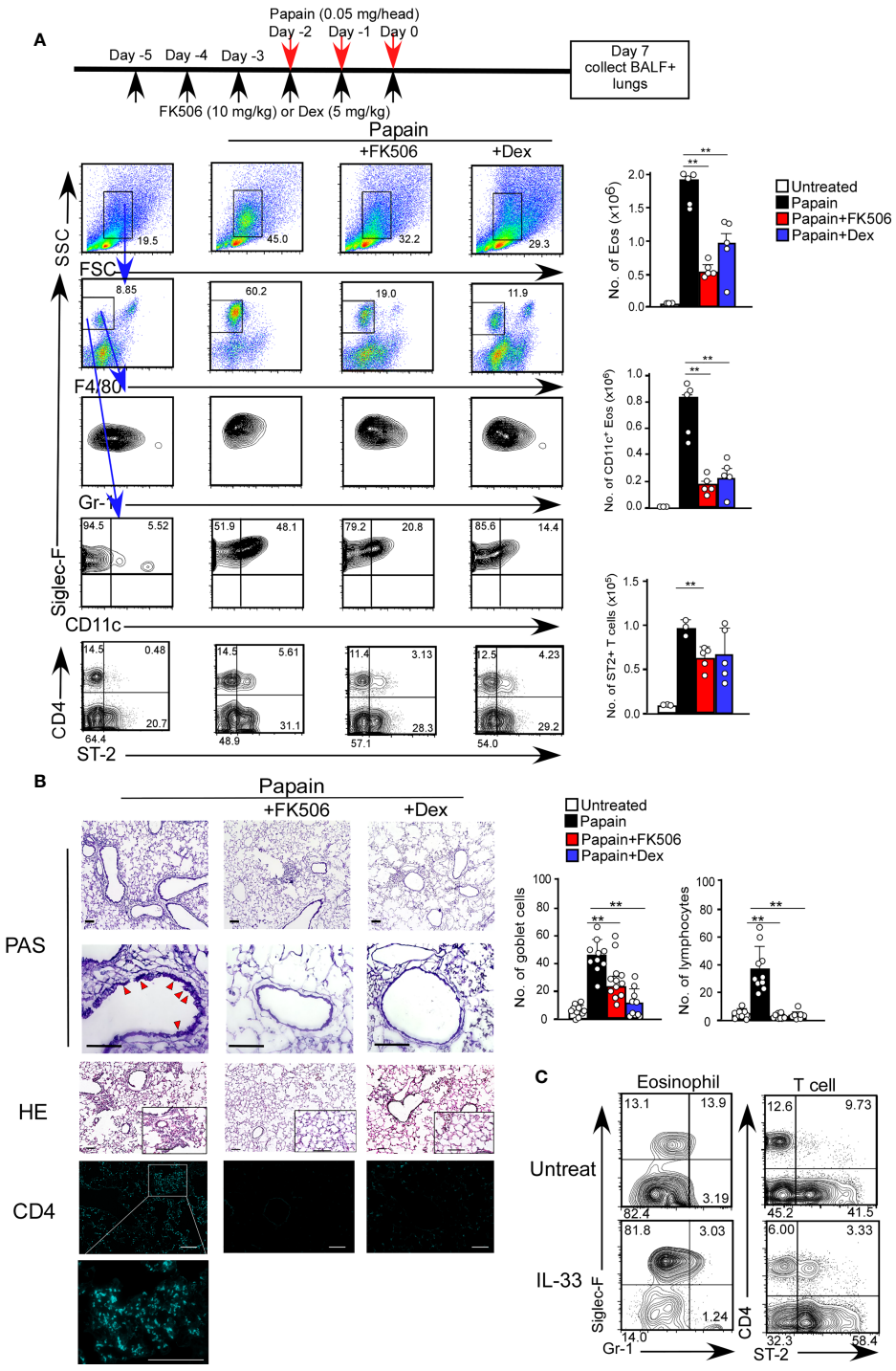

FIGURE 2
FK506 inhibited protease allergen-induced T cell-mediated airway inflammation. **(A)** The papain and FK506 or Dex treatment protocol is presented by the schematic diagram (top). Lung cells from papain-administrated mice (black bars, n=5) treated with FK506 (red bars, n=5) or Dex (blue bars, n=5) were harvested on day seven. **(B)** PAS$^+$ Goblet cells (top: low magnification; second from top: high magnification) and Eos (HE, second from bottom), and CD4$^+$ T cells (bottom, right blue) were stained in lung sections at day seven, and were analyzed by fluorescence microscopy (low magnification, x20; high magnification, x100; Scale bars, 100 μm). Goblet cells and lymphocytes were counted in 200 μm × 200 μm regions in each lung section (bar graphs, n=10). Red arrow heads indicate mucus glycans positive goblet cells. **(C)** The mice were administrated with mrIL-33 (1 μg/head/day). Lung cells were harvested on day four, and the proportions (left flow profiles) and cell numbers (right graph) of Siglec-F$^+$ and CD11c$^+$ Eos and ST-2$^+$CD4$^+$ T cells were assessed. Bars represent means; **p<0.01 using unpaired Mann-Whitney U-tests. All error bars represent SEM. FACS and section data are representative of three experiments.

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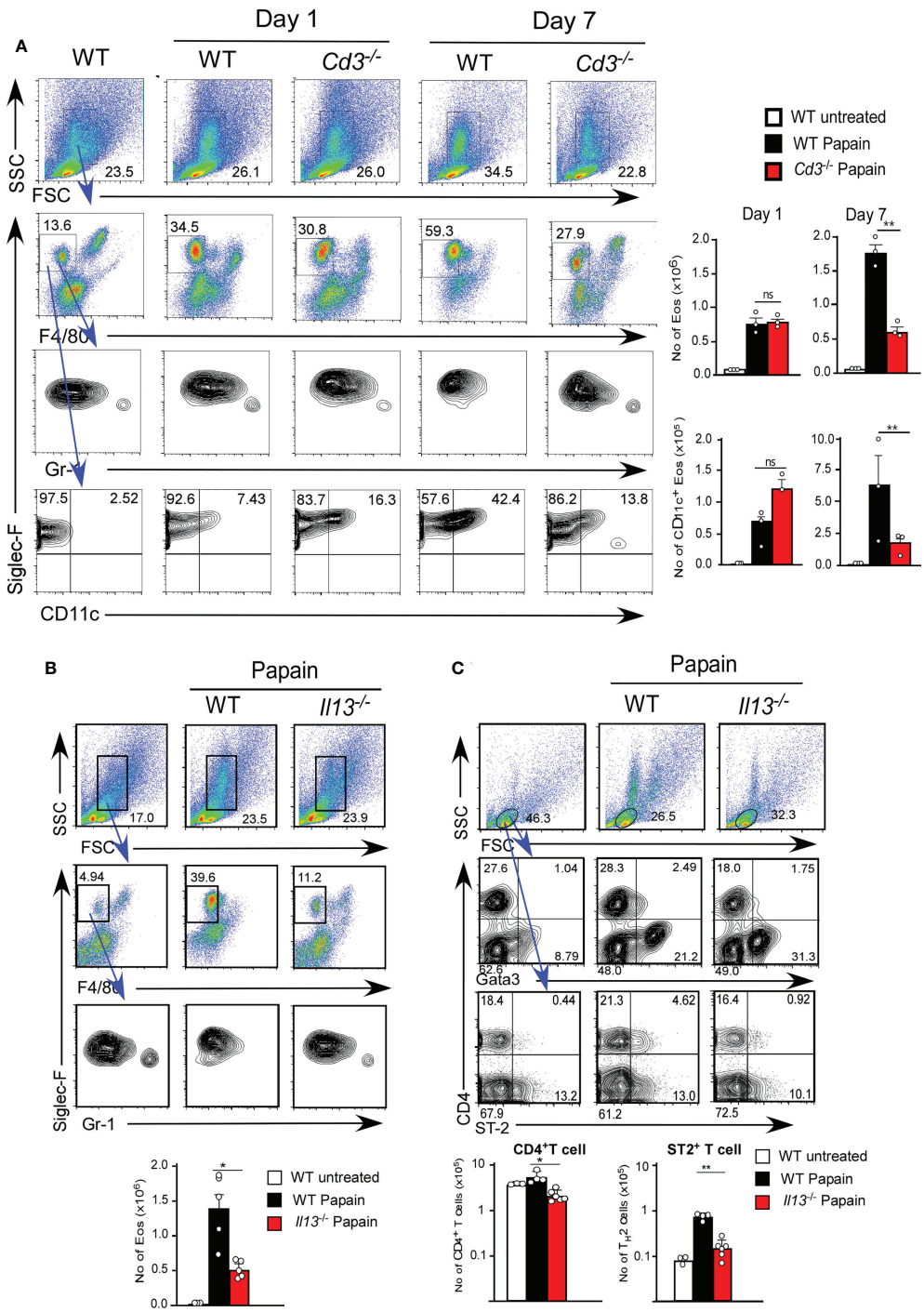

FIGURE 3
(A) Cd3-sufficient (WT, n=3) or -deficient (n=3) mice were administrated with papain. Lung cells were harvested on day one or seven after the final papain injection, and the proportions and cell numbers of Eos and T cells were assessed. **(B, C)** Il13-sufficient (WT, n=5) or -deficient (n=5) mice were administrated with papain. The proportions (left flow profiles) and numbers (right graphs) of Eos **(B)** and ST-2+CD4+ T cells **(C)** were assessed at seven days. Bars represent the means; not significant (ns), *p−0.05, **p−0.01 using unpaired Mann-Whitney U-tests. All error bars represent the SEM. Data are representative of three **(A, B)** or two **(C)** experiments.

(Figures 3A). These results indicate that T cells play a critical role in the continuous activation of eosinophils, supporting our hypothesis that the infiltration of T1/ST2$^+$ CD4 T cells contributes to the persistence of papain-induced airway inflammation.

IL-13 secreted by ILC2s is thought to be necessary for the development of T_H2 cells by modifying DC2 function (6). We next tested whether IL-13 plays a role in T1/ST2$^+$ CD4 T cell-dependent eosinophil attraction in IL-13-deficient mice (*Il13*$^{Tomato/Tomato}$). IL-13-deficient mice exhibited a marked reduction in papain-induced T1/ST2$^+$ CD4 T cell attraction along with a decrease in the persistent infiltration of eosinophils (Figure 3B). T cells that emerged in the lung after papain treatment expressed the transcription factor GATA3, which is predominantly expressed by T_H2 cells (Figure 3C). These results suggested that the T1/ST2$^+$ CD4 T cells constitute a subset of T_H2 cells. Therefore, we speculate that T1/ST2$^+$ T_H2 attraction into the lung controls the persistence of papain-induced airway inflammation and that FK506 inhibits T_H2 cell attraction by impairing ILC2 activation.

Inhibitory effect of FK506 on ILC2 activation

To examine the effect of FK506 on ILC2 activation, we performed RNA-seq analysis of lung ILC2 cells from mice nasally administered papain with or without FK506. Although FK506 treatment had no effect on the total number of ILC2s in the lungs (Figure 4A), FK506 treatment inhibited 1,300 genes whose expression increased >2-fold in response to papain. These FK506-sensitive genes included several ILC2 signature genes, such as arginase 1 (*Arg1*), *Gata3*, *Tox*, *Il13*, *Klrg1*, and *Il1r1* (Figures 4B–D; Supplementary Figure 1). These results suggest that the inhibitory effect of FK506 on T_H2 cell attraction is due to the inhibition of IL-13 and ILC2 activation in the papain-induced asthmatic responses. To investigate the effect of FK506-mediated suppression on papain-induced ILC2 activation, we measured the protein expression of ILC2-derived cytokines. Papain administration enhanced the production of GM-CSF and several type 2 cytokines (Figures 5A, B). Interestingly, papain, but not IL-33, induced detectable levels of IL-4 in activated ILC2 cells (Figure 5B). FK506 markedly inhibited the production of GM-CSF, IL-5, and IL-13 in papain-activated ILC2 cells (Figure 5A). These results suggested that the FK506-mediated inhibition of T_H2 infiltration could be due to the suppression of IL-13, and that FK506 possibly inhibited eosinophil activation by suppressing GM-CSF and IL-5 expression.

The next question concerns the role of FK506-sensitive calcium signals in ILC2 activation, which lead to eosinophil accumulation in the lungs. It has been reported that AEC-derived IL-33 activates ILC2s, IL-25, TSLP, basophil-derived IL-4, neuropeptides, and lipid mediators, including leukotrienes (LTs) (24–27), and that LTs provide calcium signals to induce IL-4 production (28). Figure 5A indicates that papain treatment triggered detectable levels of IL-4 in ILC2 cells. Therefore, we examined LT synthesis as a stimulus for ILC2 activation in this study. Naïve ILC2 cells were stimulated with cysteinyl leukotriene B_4 (LTB_4), C_4 (LTC_4), D_4 (LTD_4), or E_4 (LTE_4) in the presence or absence of FK506. ILC2 cells were also stimulated with IL-33 or PMA + ionomycin as prototypic calcium-independent or -dependent stimuli. LTB_4 provides an activation signal to induce GM-CSF, IL-5, and IL-13 production in ILC2 cells. FK506 markedly inhibited cytokine production caused by PMA+ionomycin and LTB_4 (Figure 5B), suggesting that FK506-sensitive calcium signals largely contributed to the production of GM-CSF and type 2 cytokines during papain-induced ILC2 activation.

We further examined transcriptomic changes in the LTB_4 pathway in basophils and type I and II alveolar epithelial cells (AEC1 and 2) (Supplementary Figure 2). RNA-seq analysis indicated that 5-LOX was constantly expressed in basophils but not in AECs. Moreover, LTB_4-synthesizing enzymes and leukotriene A_4 (LTA_4) hydrolase were consistently expressed in AEC1, AEC2, and basophils. FK506 inhibited the papain-induced LTA_4 hydrolase expression in AEC1, whereas their expressions in AEC2 and basophils were resistant to FK506 (Figure 5C). Moreover, FK506 seemed to be sensitive in the expression of receptors against LTB_4 in ILC2 cells, suggesting that LTB_4 signaling is a target of the FK506-mediated inhibition of ILC2 activation. In addition, we performed IPA pathway analysis of the AEC1, AEC2, and basophil transcriptome data but were unable to uncover other pathways involved in ILC2 activation (Supplementary Figure 2). Thus, we conclude that the LTB_4 pathway might be important for ILC2 activation pathway in papain-induced allergic responses and that it constitutes a putative target of the FK506-mediated inhibition of these responses. However, more studies should be conducted in order to fully validate this hypothesis.

FK506 did not inhibit IL-33-induced eosinophil attraction

Tissue damage to the airway epithelium caused by papain-mediated protease activity allows the release of IL-33, which promotes ILC2 activation. Interestingly, epithelial-derived IL-33, which is expressed explicitly in AEC2 cells, was constantly expressed at high levels irrespective of FK506 treatment (Supplementary Figure 1). However, the levels of the IL-33 receptor (*Il1rl1*) in ILC2 cells were reduced by FK506 (Figure 4C). Therefore, we assumed that the pathways downstream of IL-33 signaling are possible targets of FK506. To evaluate this, we investigated the effects of FK506 on IL-33-induced airway inflammation. The intranasal administration of

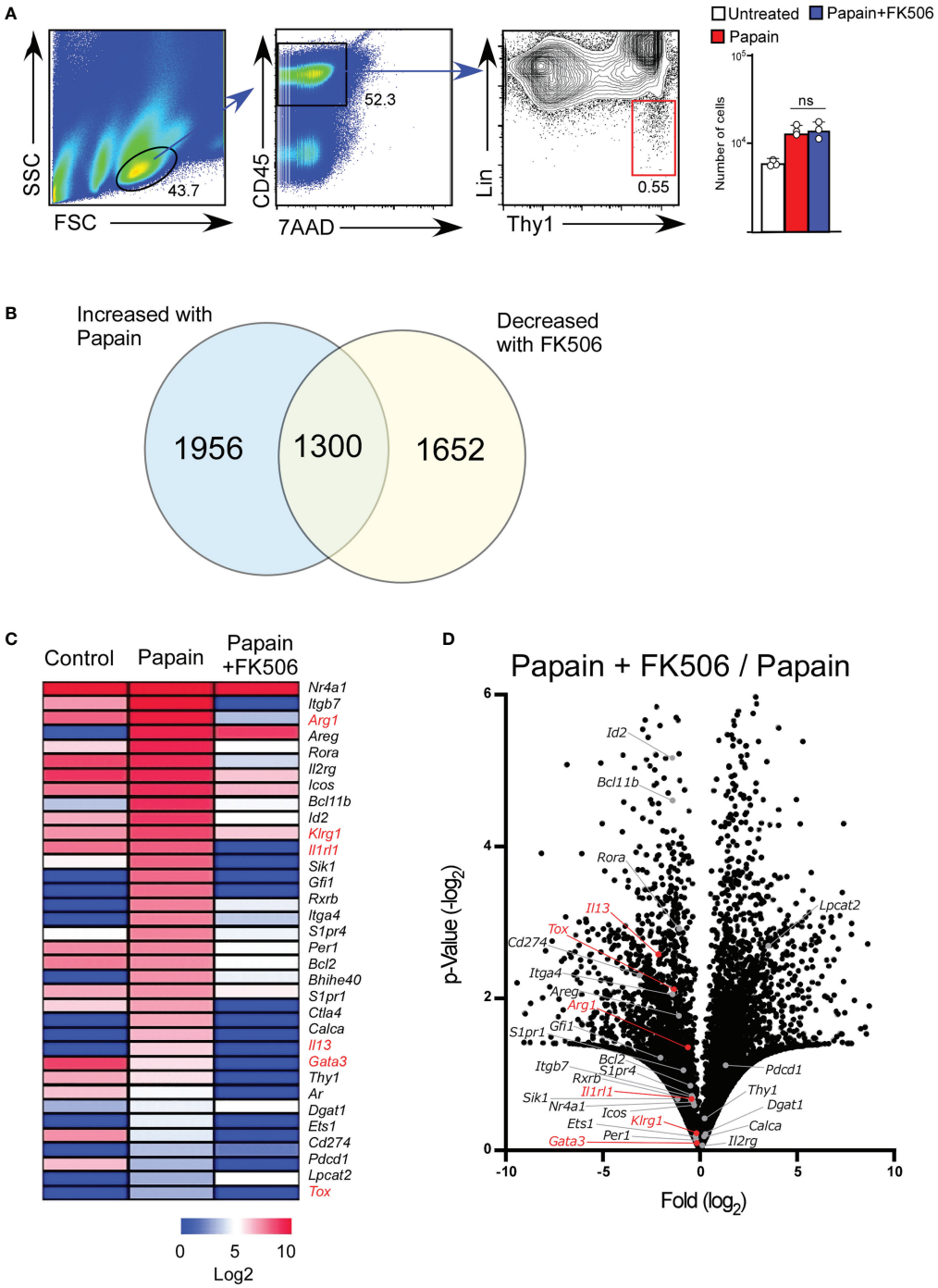

FIGURE 4
FK506 inhibits the papain-induced activation signal of ILC2 cells. Total RNA was isolated from lung ILC2 cells on day one after papain administration. We compared the results with and without FK506 treatment, as shown in Figure 1. RNAseq libraries were sequenced using the HiSeq platform. **(A)** Gating strategy and the number of lung ILC2 cells (untreated, n=3; papain-administered with or without FK506 treatment, n=3 each). CD45$^+$ lineage marker (Lin)-Ty1$^+$ ILC2 cell populations are marked with a red square. **(B)** Venn diagram of genes whose expressions were increased by papain (>2-fold) and decreased by FK506 (<2-fold). **(C)** Heat maps representing the FPKM values of ILC2 signature genes in lung ILC2 cells. The data indicate untreated (control) and papain-administered mice with (Papain + FK506) or without (Papain) FK506. **(D)** Volcano plots represent the fold-change expression (horizontal axis) and p-values (vertical axis) in the papain versus papain + FK506 comparison. ILC2 signature genes are shown in gray (n=3). ILC2 activation marker genes (Arg1, Gata3, Il13, Tox, Klrg1, and Il1rl1) are highlighted in red. Data are representative of four mice. Bars represent the means; not significant (ns) using unpaired Mann-Whitney U-tests.

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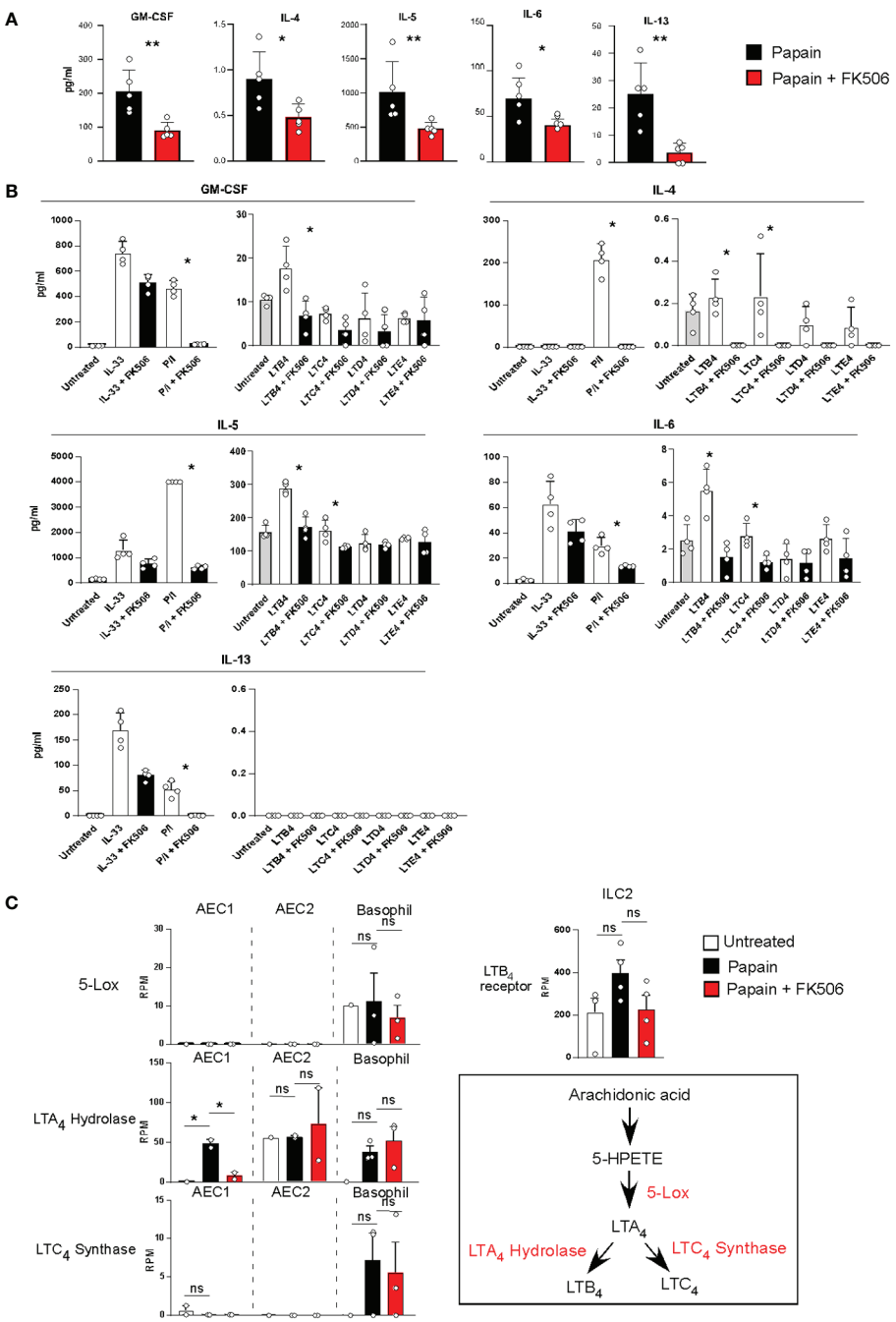

FIGURE 5
(A) Lung ILC2 cells were isolated from papain-administrated mice treated with (n=5) or without (n=5) FK506 and were cultured in the presence of rIL-7 for 12 h. Cytokine and chemokine levels in the cell supernatants were measured using MAGPIX System. **(B)** Pooled ILC2 cells were isolated from the lung of 20 unstimulated mice. Ten thousand cells were cultured on a 96-well plate in the presence of rIL-7 for 40 h. The cells were activated with LTs, IL-33, or PMA + ionomycin treated with (n=5) or without FK506 (n=5). Levels of type 2 cytokines were assessed as described above. **(C)** The left panel indicates the expression of lipid mediator genes in AEC1 cells (n=3), AEC2 cells (n=3), and basophils (n=3). The right-top panel indicates the expression of the LTB4 receptor in lung ILC2 cells as described in Figure 4. The right-bottom panel represents the biosynthetic pathway of LTB4. Bars represent the means; not significant (ns), *p−0.05, **p−0.01 using unpaired Mann-Whitney U-tests. All error bars represent the SEM.

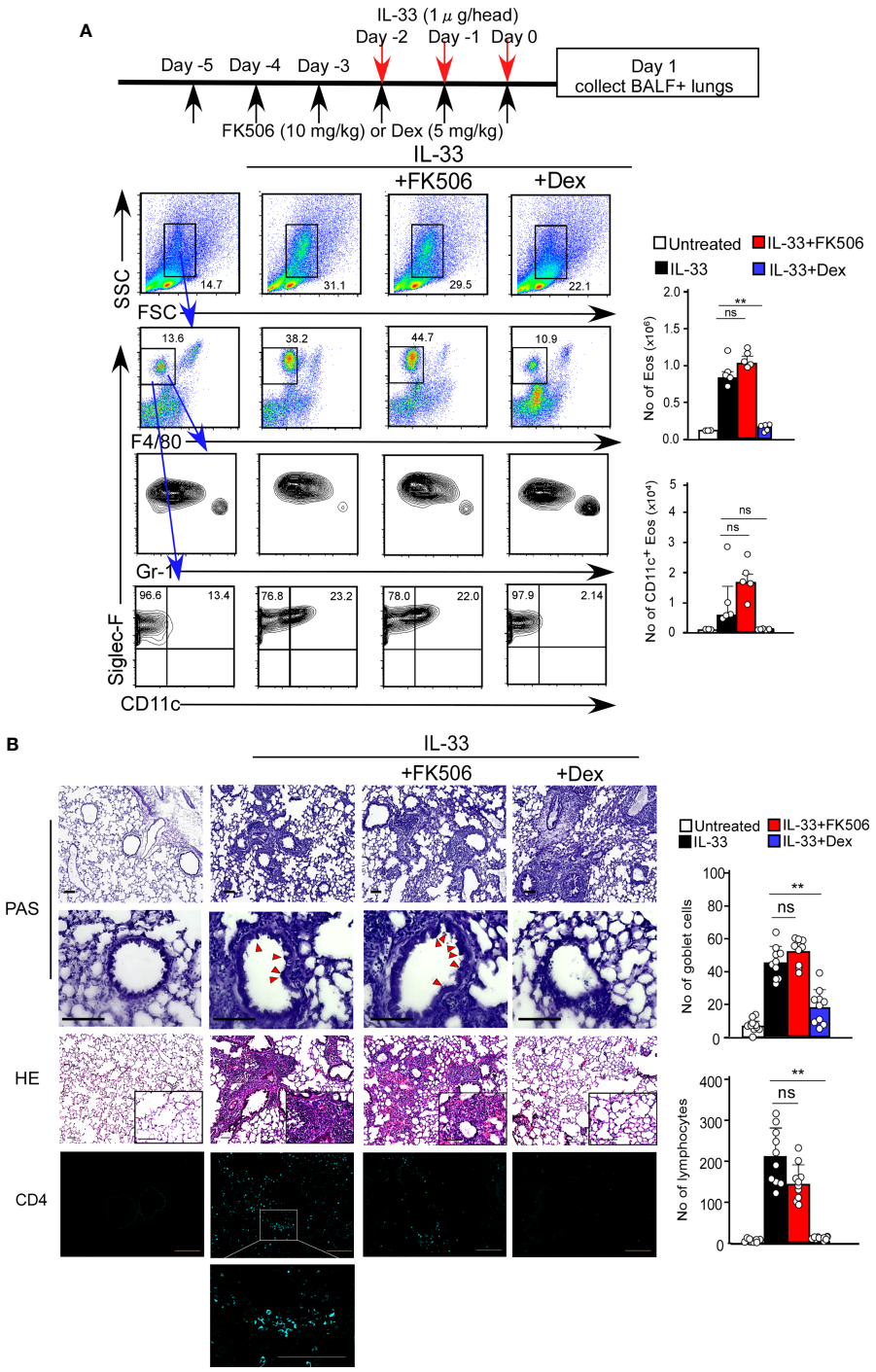

FIGURE 6
(A) The IL-33 and FK506 or Dex treatment protocol is presented by the schematic diagram (top). IL-33-administered mice (black bars, n=5) were treated with FK506 (red bars, n=5) or Dex (blue bars, n=5). Lung cells were harvested on day one after the final papain administration. The data indicated the percentages (left flow data) and the numbers (right graphs) of Eos. **(B)** PAS (top: low magnification; second from top: high magnification), HE (second from bottom) and CD4 (bottom, right blue) staining of the lung sections, and were analyzed by fluorescence microscopy (low magnification, x20; high magnification, x100; Scale bars, 100 μm). Goblet cells and lymphocytes were counted in 200 μm × 200 μm regions in each lung section (bar graphs, n=10). Red arrow heads indicate mucus glycans positive goblet cells. Bars represent the means; not significant (ns), **p−0.01 using unpaired Mann-Whitney U-tests. All error bars represent the SEM. Data are representative of three experiments.

IL-33 resulted in the marked accumulation of activated eosinophils, even in the absence of papain (Figure 6A). In addition, IL-33 promoted the hyperplasia of goblet cells and the attraction of T1/ST2$^+$ T_H2 cells (Figure 6B). Dexamethasone attenuated IL-33-induced airway inflammation; however, FK506 failed to inhibit IL-33 function (Figures 6A, B). These results suggest that FK506 mainly targets type 2 inflammation, which is controlled by the IL-33-independent activation of ILC2 cells.

Discussion

FK506, which targets calcium signaling, is a promising therapeutic agent for airway inflammatory diseases (21, 29, 30). Here, we demonstrated that FK506 dampened the induction of an asthmatic inflammatory response by inhibiting two pathways: the activation of lung ILC2 cells and the accumulation of T1/ST2$^+$ T_H2 cells. In the T cell-independent induction phase, the calcium signal of ILC2 cells was a primary target of FK506. In contrast, the induction of T_H2 cells was the second target at later responses, which migrate into the lung and persist during airway inflammation. Our analyses of a cysteine protease allergen-induced mouse model of airway inflammation demonstrated that FK506 is a potent regulator of the induction and persistence of the asthmatic inflammatory response.

FK506 inhibits T cell activation by targeting NFAT activation *via* calcium signaling and is widely used as an immunosuppressive agent (31). In this study, we showed that FK506 targets T cell migration into the lungs. T_H2 cells are essential for the persistence of airway inflammation induced by administration with cysteine protease allergen, and IL-13 is a crucial cytokine in controlling the recruitment of T1/ST2$^+$ and GATA3$^+$ T_H2 cells to the inflammatory site. The induction of T_H2 cells is critical for the persistence and exacerbation of airway inflammation (11, 12). Thus, FK506 is a potent inhibitor of the IL-13-dependent T_H2 cell recruitment induced by the nasal administration of a protease allergen.

On the other hand, FK506 was also effective in inhibiting eosinophilia during the induction phase, which is mainly controlled by ILC2 cells. Recently, Kandikattu K et al. reported that FK506 can reduce eosinophil infiltration by downregulation of calcineurin activity in the lung tissue from Aspergillus fumigatus challenged mice (23). However, eosinophil was not a direct target of FK506 in their model. Our data indicated that ILC2s were a target of FK506 in the papain induced asthmatic responses. It has been reported that FK506-sensitive calcium signaling is dispensable for IL-33-dependent ILC2 activation (32). Indeed, our data indicated that IL-33-induced airway inflammation was FK506-resistant (Figure 6). Previous evidence indicated that other calcium-dependent signaling molecules, lipid mediators, and NMU can also control ILC2 cell activation (24, 32). Our data suggest that the LTB_4 signal could be a possible target of FK506 in the ILC2 activation process. Locksley et al. reported that cyclosporine A, another calcineurin inhibitor, reduced IL-13 production in cultured ILC2 cells stimulated with LTB_4 (24). Therefore, FK506-sensitive calcineurin-mediated ILC2 activation is critical for protease-mediated airway inflammation.

Eosinophils are the primary effector cells involved in asthmatic airway inflammation (10, 33). Several previous reports have indicated that blocking GM-CSF signaling promotes the inhibition of type 2 inflammatory responses (34–38). Here, we found that FK506 suppressed GM-CSF production in ILC2 cells. Thus, the inhibition of ILC2-derived GM-CSF may be a possible mechanism of the FK506-mediated suppression in the protease-mediated airway inflammation during the induction phase.

In conclusion, we demonstrated that T_H2 and ILC2 cells could be therapeutic targets for FK506 in type 2 airway inflammation. FK506 inhibits the differentiation of T_H2 cells, which causes chronic inflammation by attenuating ILC2 function. Since ILC2 and T_H2 cells are involved in allergic airway inflammation, such as asthma, our results strongly support the potential clinical value of FK506 for these type 2 inflammatory diseases. The inhibitory mechanism of FK506 in a cysteine protease allergen-induced allergic mouse model sheds new light on future therapeutic strategies for asthma.

Methods

Mice

C57BL/6Jcl mice were purchased from CLEA (Meguro, Tokyo, Japan). Six- to ten-week-old female mice were used in the experiments. *Cd3e*$^{-/-}$ and *Il13*^{tomato} mice were kindly provided by Dr. Bernard Malissen (Aix Marseille Université, Marseille, France) and Dr. Andrew NJ McKenzie (MRC Laboratory of Molecular Biology, Cambridge, UK) (39, 40).

All transgenic mice were obtained from a C57BL/6 background. All mice were maintained under specific pathogen-free conditions, and animal care was performed according to the guidelines of the RIKEN Yokohama Institute.

Cysteine protease allergen-induced and IL-33-induced allergic mouse model

Mice were intranasally administered with papain (50 μg/head/day; Sigma-Aldrich, St. Louis, MO, USA) or IL-33 (1 μg/head/day; Biolegend, San Diego, CA, USA) for three days. The lungs and bronchoalveolar lavage (BAL)s of the treated mice were harvested on days 1 and 7. The harvested lung tissues were roughly chopped with a Gentle MACS Dissociator (Miltenyi Biotec) and digested with 5 mL of HBSS (Thermo Fisher, Waltham, MA, USA) containing DNase I (75 μg/mL) and

collagenase D (400 U/mL) for 30 min at 37°C. The lung homogenate was ground with a Gentle MACS Dissociator, passed through a 100-μm cell strainer (BD Biosciences, Franklin Lakes, NJ, USA), and fractionated with 30% Percoll (GE Healthcare, Uppsala, Sweden). The cell pellet was then treated with an RBC lysis buffer (Biolegend, San Diego, CA, USA) and suspended in MojoSort buffer (Biolegend). After the combining of BAL and lung isolated cells, eosinophils were analyzed using anti-Siglec-F, anti-Gr-1, and anti-CD11c antibodies after eliminating alveolar macrophages with anti-F4/80 antibodies (Figure 1).

For chemical treatment, the mice were intraperitoneally treated with FK506 (0.2 mg/head/day; Cayman Chemical, Ann Arbor, MI, USA) or Dexamethasone (0.1 mg/head/day; Sigma-Aldrich, St. Louis, MO, USA) for six days. These mice were then administered with papain intranasally in the last three days.

Histology

The lungs were fixed with paraformaldehyde (4%) and frozen in OCT compound (Sakura Finetek, Tokyo, Japan). Sections (5 μm) were stained with an HE or PAS Staining Kit (Muto Pure Chemicals, Tokyo, Japan), or anti-CD4 antibody (clone: RM4-5; 1:50, Biolegend, 100506). Images were acquired using a BZ-X700 microscope (Keyence). Two regions of interest (ROIs) were set on each section, and the numbers of acidic mucus-positive goblet cells and infiltrating lymphocytes were counted.

Flow cytometry

Cell staining was performed using antibodies against B220 (clone: RA3-6B2; used at 1:500, Biolegend, 103227), CD3e (clone:145-2C11; 1:500, Biolegend, 100304), CD4 (clone: GK1.5; 1:500, Biolegend, 100423), CD5 (clone:53-7.3; 1:200, Biolegend, 100604), CD8a (clone:53-6.7; 1:500, Biolegend, 100704), CD11b (clone: M1/70; 1:200, Biolegend, 101204), CD11c (clone: N418; 1:200, Biolegend, 117304), CD45.2 (clone:104; 1:500, eBioscience, 11-0454-85), CD49b (clone: DX5; 1:200, Biolegend, 108904), F4/80 (clone: BM8; 1:200, Biolegend, 123106), FceR1 (clone: Mar1; 1:200, Biolegend, 134318), Gr-1 (clone: RB6-8C5; 1:500, BD Biosciences, 553124), NK1.1 (clone: PK136; 1:300, Biolegend, 108704), Siglec-F (clone: E50-2440; 1:400, BD Biosciences, 552126), Thy1.2 (clone:30-H12; 1:500, BD Biosciences, 105324), Podoplanin (clone:8.1.1; 1:100, Biolegend, 127410), and Ter119 (1:200, eBioscience, 13-5921-82). Flow cytometric analysis and cell sorting were performed using FACSCalibur and FACSAria III systems (BD Biosciences), and data were analyzed using FlowJo software (BD Biosciences). Doublet cells were excluded by FL-2A/FL-2H plots, then FSC/SSC plots were used to narrow down eosinophil or T cell populations followed by further gating with Siglec-F/Gr-1 or Siglec-F/CD11c (for eosinophils), or CD4/ST-2 (for T_H2 cells) plots (Supplementary Figure 3).

RNA-seq analysis

Total RNA was isolated from freshly sorted lung epithelial cells, basophils, and ILC2 using the TRIzol reagent. The 3' mRNA-seq Library Prep Kit (Lexogen, Vienna, Austria) was used for constructing sequencing libraries. RNA libraries were prepared for sequencing using the standard Lexogen 3' QuantSeq protocols. After sequencing using the HiSeq 1500 platform (Illumina, San Diego, CA, USA), sequenced reads were trimmed for adaptor sequences, masked for low-complexity or low-quality sequences, and mapped to the whole mouse genome (mm10) using STAR 2.7.0 c (41).

Cytokine analysis in ILC2 cells

Lineage-positive lung $CD45^+$ cells were eliminated using lineage markers (CD3e, CD4, CD5, CD8a, CD11c, CD19, F4/80, Ly-6G, and NK1.1), and $Thy1.2^+$ cells were isolated as ILC2 cells. To assess the levels oILC2-derived cytokines in papain-treated mice, isolated ILC2 cells were cultured in RPMI medium containing 10 ng/mL of recombinant murine IL-7 (rIL-7) (Peprotech, Cranbury, NJ, USA) for 12 h, and cytokine and chemokine levels in the supernatant were measured using a MAGPIX Multiplexing System (Luminex, Austin, TX, USA) and a MLLIPLEX Mouse High Sensitivity T Cell Panel (Merck, Darmstadt, Germany).

To investigate the role of calcium signaling in ILC2 cells, isolated ILC2 cells were stimulated with 10 ng/mL of recombinant murine IL-33 (Biolegend), 30 ng/mL of PMA (Sigma-Aldrich), 500 ng/mL of ionomycin (Sigma-Aldrich), or 10 nM of LTB_4, LTC_4, LTD_4, or LTE_4 (Cayman Chemical) in the presence of 10 ng/mL of rIL-7 (Peprotech) for 40 h. Stimulation was performed with or without FK506 (1000 nM). Cytokine levels were determined as previously described (25).

Statistical analyses and reproducibility

Statistical comparisons between groups were performed using Prism software version 8.0.2 (Graph Pad Software, San Diego, CA, USA). Data are presented as the mean ± SEM. Statistical analyses were performed using the Mann-Whitney U test (*$p<0.05$, **$p<0.01$).

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and

accession number(s) can be found in the article/ Supplementary Material.

Ethics statement

RIKEN Yokohama Institutional Animal Care Committee reviewed and approved the animal study.

Author contributions

MK designed and conceptualized the research; CT, NS, YS, and KM performed the mouse experiments; CT and SK performed the histological analysis; CT, NS, and KM performed RNAseq analysis; KM, HM, YM, and MK prepared the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

HM and YM are employees of Maruho Co. Ltd.

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

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.1014462/full#supplementary-material

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Burn-injured skin is marked by a prolonged local acute inflammatory response of innate immune cells and pro-inflammatory cytokines

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The systemic and local immune response in burn patients is often extreme and derailed. As excessive inflammation can damage healthy tissues and slow down the healing process, modulation of inflammatory responses could limit complications and improve recovery. Due to its complexity, more detailed information on the immune effects of thermal injury is needed to improve patient outcomes. We therefore characterized and quantified subsets of immune cells and mediators present in human burn wound tissue (eschar), sampled at various time points. This study shows that after burn injury, the number of immune cells were persistently increased, unlike the normal wound healing process. There was an immediate, strong increase in neutrophils and a moderate increase in monocytes/macrophages and lymphocytes, especially in the second and third week post burn. The percentage of classical ($CD14^{high}CD16^{-}$) monocytes/macrophages demonstrated a steady decrease over time, whereas the proportion of intermediate ($CD14^{high}CD16^{+}$) monocytes/macrophages slowly increased. The absolute numbers of T cells, NK cells and B cells increased up to week 3, while the fraction of γδ T cells was increased only in week 1. Secretome profiling revealed high levels of chemokines and an overall pro-inflammatory cytokine milieu in burn tissue. The local burn immune response shows similarities to the systemic immune

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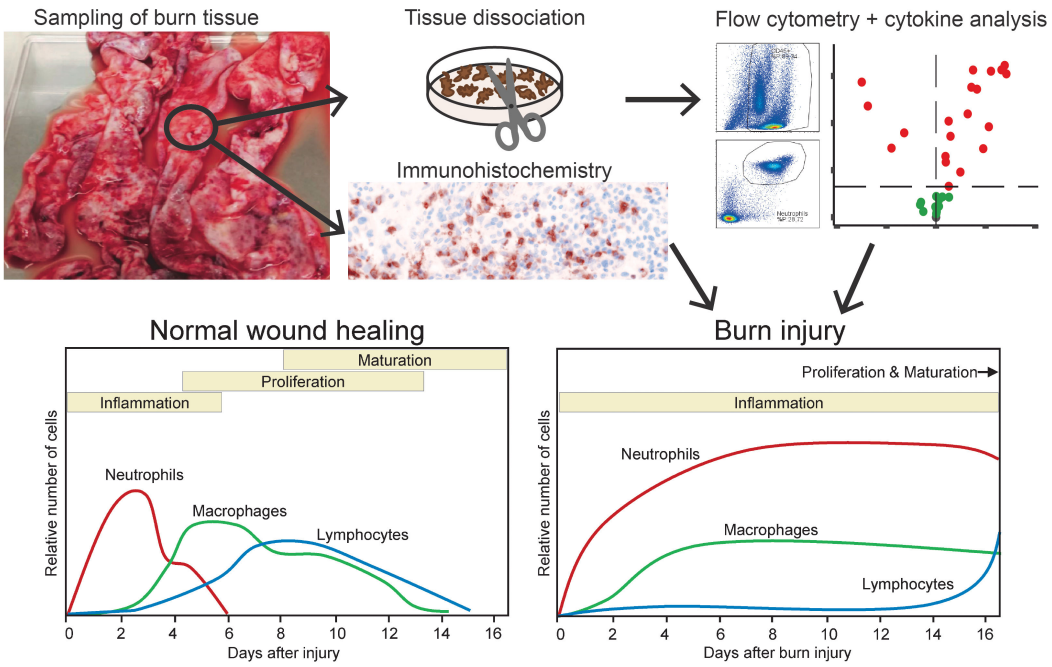
reaction, but differs in neutrophil maturity and lymphocyte composition. Altogether, the neutrophil surges, high levels of pro-inflammatory cytokines and limited immunosuppression might be key factors that prolong the inflammation phase and delay the wound healing process in burns.

KEYWORDS

cell isolation, immune cells, flow cytometry, neutrophils, lymphocytes, macrophages, inflammation, burn wound tissue



GRAPHICAL ABSTRACT
Graphical overview of the study. Top part shows the investigation of burn wound tissue using flow cytometry, immunohistochemistry and cytokine analysis. Bottom part shows the relative response of immune cells during normal wound healing (as described in literature) and after burn injury (as shown by the study results) (1).

Introduction

Burn injury is often accompanied by an extensive, derailed immune response in both burn wound tissue and peripheral blood (2, 3). Regardless of infection, burn patients generally show signs of systemic inflammation caused by high levels of cytokines and danger signals that originate from damaged tissue (4, 5). Necrotized and inflamed tissue stimulates the immune system to recruit acute phase immune cells to the affected site (3, 6, 7). Fibroblasts and keratinocytes surrounding the wound site and infiltrating leukocytes release a storm of cytokines, chemokines and growth factors that initiate the inflammation phase (8).

Typically during wound healing, neutrophils and macrophages with a pro-inflammatory (i.e. M1) phenotype will migrate into the wounded skin to remove debris and prevent bacterial colonization (9). Within days, wound neutrophils will disappear through apoptosis and macrophages will differentiate into a state that supports wound healing (i.e. M2 phenotype) (10). Generally within week after injury, lymphocytes will infiltrate the wound site to orchestrate tailored pathogen-eliminating and immune cell regulating

responses (11). The reduction, transition and control of immune cells are crucial for dampening of the inflammatory response and for the establishment of a healthy wound healing process. After burn injury however, the immune system can be overactive and is then likely to cause damage to surrounding tissues, delay wound healing and contribute to the severity of scarring (3, 7).

Burn patients who experience persistent inflammation might benefit from immune suppressive treatment, however at the same time they are at risk of contracting infections such as pneumonia or cellulitis, caused by opportunistic bacteria (12). Therefore, innovative and precise interventions that modulate the immune response could be crucial in the relief of secondary illnesses while improving wound healing and preventing infection. Still, there is only little information on the immune response after burn injury and how exactly it differs from normal wound healing, mainly due to its complexity and variation among cases (e.g. burn size, depth and cause) and burn patients (e.g. age, sex and co-morbidities) (13). Moreover, present evidence on the processes that underlie burn injury originates mostly from animal research (14), which is only partially translatable to the human situation (15). We previously showed that in blood from severely burned patients, there was an extreme increase in innate immune cells and pro-inflammatory cytokines (8). In this longitudinal study, we investigated immune cells and soluble factors present in burn wound tissue (eschar) that was surgically debrided as part of standard treatment (16). A better understanding of the immune reactions to burn injury will facilitate the design of improved and more targeted treatment approaches for trauma-induced immune dysfunction.

Materials and methods

Sample collection

Burn wound tissue (eschar) from patients of all ages and thermal burn causes who underwent eschar debridement as part of their treatment at the Burn Center of the Red Cross Hospital in Beverwijk, the Netherlands. Healthy skin samples were obtained from adult patients who underwent cosmetic surgery (abdominoplasty or elective) at the Department of Plastic and Reconstructive Surgery of the Red Cross Hospital. Tissue samples were collected in the period between February 2019 and December 2021. Consent for the use of residual samples was received through the opt-out protocol of the Red Cross Hospital, in accordance with the national guidelines (https://www.coreon.org/). Subjects were informed of this procedure and were able to withdraw at any point. After surgical removal, tissue samples were stored in RPMI 1640 (Gibco, Paisley, UK) containing 1% penicillin and streptomycin (Gibco) as soon as possible to increase cell survival (17, 18). Samples were stored overnight at 4 °C and processed the following morning. Subject and sample characteristics are shown in Supplementary Table 1.

Single cell isolation

This protocol was based on the immune cell isolation procedure from He et al. (19). Biopsies were taken from viable areas of the burn tissues, i.e. white or red areas with bleeding spots and not blackened or leathery areas. Approximately 600 mg of tissue was used per cell isolation for flow cytometry (FCM). Tissue samples were cut into smaller pieces and subsequently divided over 2 C-tubes (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) containing 5 mL of RPMI 1640 containing 1% penicillin and streptomycin. C-tubes were placed on a tissue dissociator (gentleMACS, Miltenyi Biotec) and program "B" was run. Hundred-fifty µL of 80 mg/mL collagenase I (Merck, St. Louis, MO, USA) in PBS (Gibco) was added and the sample was incubated for 1 h in a shaking water bath at 37 °C. After incubation, the C-tube was placed on the tissue dissociator to run program "B". Samples were passed through a 500 µm and 40 µm cell strainer (pluriSelect, Leipzig, Germany) to obtain a single cell suspension. Suspensions were centrifuged for 10 min at 450 × *g*, and supernatant was discarded. The cell pellet was resuspended in erythrocyte lysis buffer (1.5 mM NH_4Cl, 0.1 mM $NaHCO_3$ and 0.01 mM EDTA in demineralized water) for 10 min at room temperature. Twenty mL of FCM buffer (PBS containing 1% BSA, 0.05% natrium-azide and 1 mM EDTA) was added and the suspension was centrifuged for 10 min at 450 × *g*. The pellet was resuspended in 5 mL of FACS buffer and cells were counted on the flow cytometer (MACS Quant Analyzer 10, Miltenyi Biotec GmbH, Bergisch Gladbach, Germany).

Supervised flow cytometry

From the single cells suspensions approximately 2.5×10^5 cells were used per staining panel. Antibodies used for FCM are displayed in Supplementary Table 2. A solution of 7-AAD (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) or propidium iodide (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) were used to calculate viability of cells. Stained cell samples were acquired on the MACS Quant Analyzer 10 and analysis was performed using FlowLogic (Inivai Technologies, Victoria, Australia). Gating strategy is shown in Supplementary Figure 1. Data was visualized using Graphpad version 5.01 (PRISM, La Jolla, USA) and R (ggplot package).

Unsupervised flow cytometry analysis

Lymphocyte (panel 1), T cell (panel 2), neutrophil (panel 3) or monocytic (panel 4) populations were gated based on FSC/SSC, CD45, CD3, CD15 in MACSQuantify 2.13.3 software (Miltenyi Biotec). Data of these sole populations were uploaded to Cytobank (20) to create Flow Self-Organizing Map (FlowSOM) clusters.

Immunohistochemistry

Kryofix (50% ethanol, 3% PEG300)-fixed paraffin-embedded 5 µm thick sections were used after deparaffinization and rehydration. Endogenous peroxidase was blocked in 1% hydrogen peroxide for 15 min at room temperature. Next, antigen retrieval for different antigens was performed. The blocking step was performed using 5% normal goat serum (Merck) diluted in PBS + 1% bovine serum albumin (BSA). Tissue sections were then incubated with the primary antibodies (Supplementary Table 3) for 1 h at RT followed by incubation with a poly-HRP-goat-anti-mouse or rabbit secondary antibody (BrightVision, VWR) for 30 min at RT. Detection of the target protein was established using 3,3′-Diaminobenzidine (BrightDAB, VWR). After immunohistochemical (IHC) DAB staining was successful, sections were counterstained with hematoxylin, dehydrated and mounted with Eukitt Mounting Medium (Merck). Percentage of MPO, CD3 or CD68 positive area was calculated using NIS Elements (Nikon Instruments Europe B.V.) and based on 3 images from representative tissue sections.

Multiplex imaging and analysis

Formalin-fixed and paraffin-embedded 5 µm sections were deparaffinized using xylene and rehydrated with ethanol and distilled water. Antigen retrieval was performed by boiling in TRIS-borate-EDTA buffer for 10 min. A multiplex staining for the detection of neutrophils and lymphocytes was performed performed using the protocol described by Rodriguez et al. (21).

Immunoassay of tissue homogenates

Frozen tissue samples of approximately 60 mg were thawed, minced into smaller pieces and further dissociated in M-tubes (Miltenyi Biotec) by adding lysis buffer (PBS containing 0.01 mM EDTA and protease inhibitor (1 tablet per 10 mL; Pierce, Thermo Scientific)) and running program "Protein_01" on a gentleMACS (Miltenyi Biotec). Debris was removed from the samples using a filter plate (Multiscreen, Merck) and samples were diluted to concentration of 12 mg tissue/mL. Luminex assay was performed according to the manufacturer's instructions (Merck KGaA). The following assay kits were used: HCYTA-60K, TGFBMAG-64K, HCYP2MAG-62K and HTH17MAG-14K. In short, 25 µL of tissue homogenate was used to determine the concentrations of 37 soluble factors, namely MCP-1 (CCL2), MIP-1α (CCL3), MIP-1β (CCL4), MIP-3α (CCL20), GROα (CXCL1), IP-10 (CXCL10), IFN-α2, IFN-γ, TNF-α, TGF-β1, TGF-β2, TGF-β3, CTACK (CCL27), RANTES (CCL5), IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8 (CXCL8), IL-9, IL-10, IL-12p40, IL-12p70, IL-13, IL-17A (CTLA-8), IL-17F, IL-18, IL-21, IL-22, IL-23, IL-33 (NF-HEV), GM-CSF, PDGF-AA, PDGF-AB/BB and VEGF-A. For TGF-β1,2,3 samples were acid-treated prior to the assay, according to the manufacturer's instructions. Mean fluorescence intensity of samples was measured with a Flexmap 3D System (Luminex Corp, Austin, USA) and concentrations were calculated using Bio-Plex Manager Software (Bio-Rad Laboratories, Veenendaal, The Netherlands). Values below the minimum of the standard were based on extrapolation of the standard curve by the software.

Statistical analyses

Distribution of the data was checked for normality using the Shapiro Wilk test. For the FCM and IHC data, differences between burn tissue and healthy skin, and between burn tissues of different time intervals after injury (PBW 1, 2, 3 and 4) were explored using the Mann Whitney U test in Graphpad version 5.01 (PRISM, La Jolla, USA). Only statistically significant differences are shown and are indicated by black asterisks (* = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$). The data was visualized using Graphpad version 5.01 (PRISM, La Jolla, USA). Levels of the soluble immune factors in burn tissue were transformed to fold change differences compared to the levels in healthy skin. P values of differences between burn tissue and healthy skin were determined using Mann Whitney U tests. We considered a p value of < 0.05 to be statistically significant. Volcano plots were created using "EnhancedVolcano" version 1.6.0 package in R version 3.6.2.

Results

Burn injury is followed by a strong local increase in granulocytes and moderate increase in monocytes and lymphocytes

Local immune effects of burn trauma were investigated in burn tissue that was debrided during routine surgical procedures (subject and sample characteristics shown in Supplementary Table 1). We selected viable sections of tissue biopsies and neglected necrotized or blackened segments to ensure the isolation of viable cells. CD45 immunohistochemical (IHC) staining showed an extreme infiltration of leukocytes ($CD45^+$

cells) in burn tissue (Figure 1A). The majority of leukocytes were viable after isolation from healthy skin (90.3% ± 6.6) and burn tissue (89.1% ± 10.5) (Supplementary Figure 2A). Flow cytometry (FCM)-based quantification revealed that the increase in leukocyte numbers was most abundant at post burn week (PBW) 2-3 (Figure 1B). As a result, the percentage of $CD45^-$ cells, which include fibroblasts, keratinocytes and endothelial cells, was lower in burn tissue from PBW 2-3 than in healthy skin (Supplementary Figure 2B). The leukocytes isolated from healthy skin consisted of approximately 25% granulocytes, 55% monocytic cells (monocytes and macrophages) and 20% lymphocytes (Figure 1C). In burn tissue from PBW 1, there were 52% granulocytes, while for the proportion of monocytic cells was 29%. The lymphocytes fraction in burn tissue was similar to healthy skin (19%). In burn tissue from PBW 2-4, the portion of granulocytes was still enlarged (55-62%), while the fraction of monocytic cells decreased further to 13-16% and the lymphocyte fraction

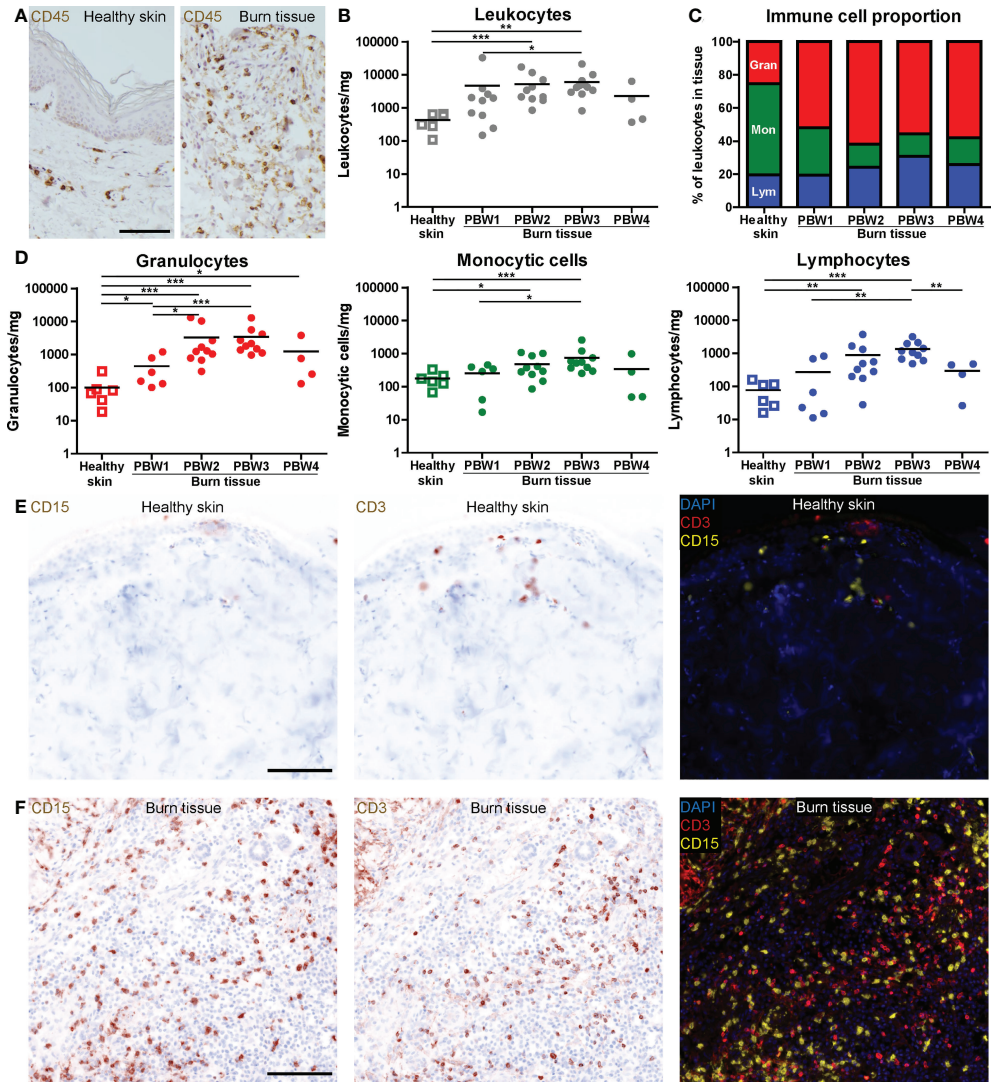


FIGURE 1
High number of immune cells infiltrate the skin as response to burn injury. **(A)** CD45 immunohistochemical DAB staining of a representative section of healthy skin and burn tissue (from 15 days post burn) (black scale bar = 100 µm). Flow cytometry-based quantification of: **(B)** Absolute number of leukocytes per mg tissue (based on side scatter and CD45); **(C)** Proportion of granulocytes (Gran), monocytic cells (Mon) and lymphocytes (Lym) in tissue (based on side scatter and CD45); **(D)** Absolute numbers of granulocytes, monocytic cells and lymphocytes per mg tissue (based on side scatter and CD45). Microscopic image of multiplex DAPI, CD15 (granulocytes) and CD3 (T cells) staining of a representative: **(E)** Healthy skin sample; **(F)** Burn tissue sample (from 25 days post burn), shown separately and as composite (black scale bar = 100 µm). P values were calculated using Mann-Whitney U statistical tests, significant differences are indicated by black asterisks: *p < 0.05; **p < 0.01; ***p < 0.001.

increased (24-31%). During PBW 1-3, absolute number of granulocytes, monocytic cells and lymphocytes rose and declined only at PBW 4 (Figure 1D). Multiplex spatial phenotyping of healthy skin and burn tissue sections using CD3 and CD15 revealed dense areas populated with granulocytes and T cells in burn tissue (Figures 1E, F).

Granulocytes in burn tissue consist mainly of activated mature neutrophils

IHC analysis of myeloperoxidase (MPO) expression, an enzyme abundantly present in azurophilic granules of neutrophils (22), showed an immediate increase in neutrophil numbers in burn tissue at PBW 1, and an even larger increase from PBW 2 onward (Figures 2A, B). This was confirmed by FCM analysis of neutrophils ($CD15^+CD16^+$ granulocytes) (Figure 2C). Eosinophils ($CD9^+CD15^+CD16^-$ granulocytes) were increased at PBW 2-3, but to a lesser extent (Supplementary Figure 2C). In both healthy skin and burn tissue neutrophils were almost exclusively $CD10^+$, a marker that is associated with neutrophil maturation (23) (Figure 2D). Only in burn tissue from PBW 1 there was a slight increase in immature ($CD10^-$) neutrophils. Activation markers CD11b and CD66b were upregulated in neutrophils at PBW 2-3 (Figures 2E, F). Self-organizing map clustering of flow data (FlowSOM) using Cytobank displayed cell populations (nodes) and clusters based on marker expression in an unsupervised manner (Figure 2G). This analysis highlights some of the burn-specific changes that occur in wound neutrophils. Burn injury caused significant differences in the percentage of neutrophils per cluster (Figure 2H). $CD11b^{low}CD14^+CD66b^-$ neutrophils (cluster 1) were decreased early after burn injury, while $CD11b^+CD66b^{low}$

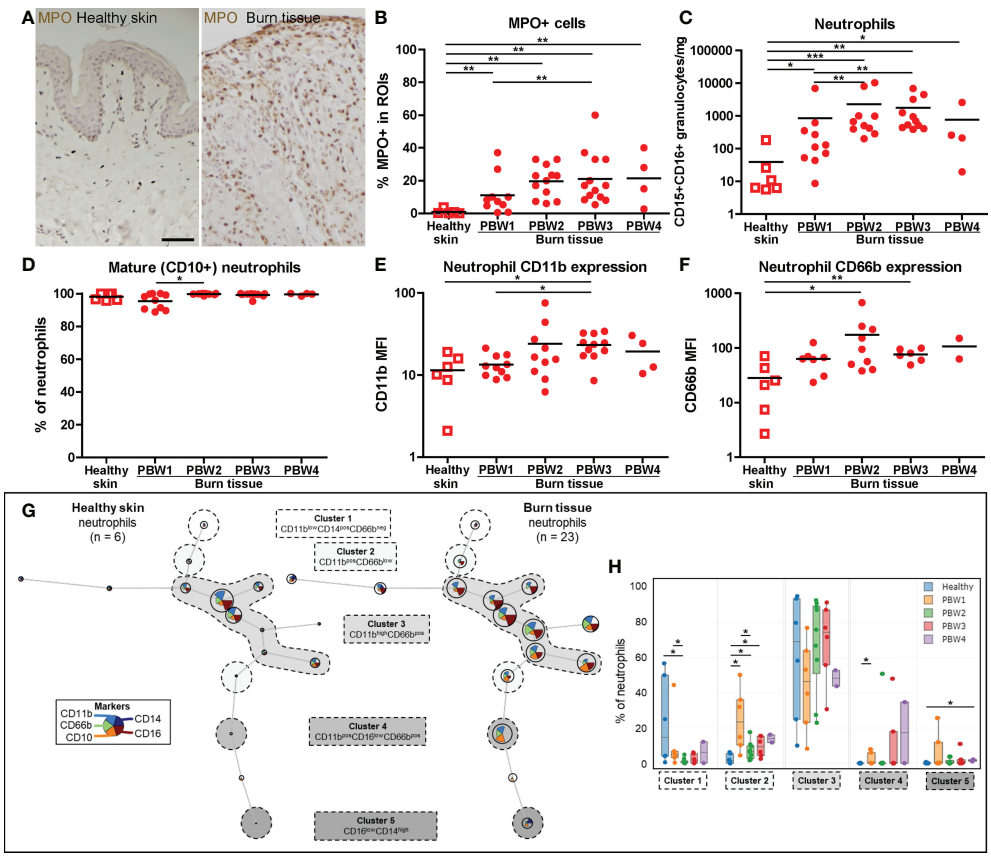


FIGURE 2
Local neutrophil response to burn injury. **(A)** Myeloperoxidase (MPO) immunohistochemical DAB staining of a representative section of healthy skin and burn tissue (from 15 days post burn) (black scale bar = 100 µm). **(B)** MPO^+ area of tissue sections. Flow cytometry-based quantification of: **(C)** Absolute number of neutrophils ($CD15^+CD16^+$ granulocytes) per mg tissue; **(D)** Percentage of $CD10^+$ (mature) neutrophils ($CD15^+CD16^+$ granulocytes); **(E)** MFI of CD11b on neutrophils ($CD15^+CD16^+$ granulocytes) in tissue; **(F)** MFI of CD66b on neutrophils ($CD15^+CD16^+$ granulocytes) in tissue. **(G)** Unsupervised clustering of neutrophil ($CD15^+CD16^+$ granulocytes) flow data from healthy skin and burn tissue, 5 clusters are highlighted. Node size represents relative size of population and node diagram shows expression level of markers. **(H)** Percentage of neutrophils within each cluster. Error bars in H show boxplot, p values were calculated using Mann-Whitney U statistical tests, significant differences are indicated by black asterisks: *p < 0.05; **p < 0.01; ***p < 0.001.

neutrophils (cluster 2) were increased. Although CD11b^{high}CD66b^{+} neutrophils (cluster 3) seemed more represented in burn tissue than in healthy skin, no significant difference was found. A small population of CD16^{low} neutrophils (cluster 4) was significantly increased at PBW 1 and the percentage of CD16^{low}CD14^{+} neutrophils (cluster 5) was significantly increased at PBW 4.

Burn injury increases macrophage numbers and affects differentiation

Macrophage differentiation was assessed by analyzing the CD14 and CD16 expression of the monocytic cell population (among which are monocytes and macrophages) using flow cytometry. In both healthy skin and burn tissue the majority of these cells expressed a classical phenotype (CD14^{high}CD16^{-}) (Figures 3A, B). In burn tissue from PBW 3, the proportion of classical monocytic cells decreased while the proportion of intermediate (CD14^{high}CD16^{+}) monocytic cells increased. Next, we analyzed the macrophages (CD68^{+} cells) within the monocytic cell population and found a steady increase in macrophages over time after burn injury (Figures 3C–E). By both IHC and FCM we could detect a significant increase in macrophages at PBW 3. Macrophage phenotype was further investigated by analyzing CD40 and CD80 expression (indicative for pro-inflammatory phenotype) and CD163 and CD206 expression (hallmarks for pro-healing) (Supplementary Figure 2D). The only significant difference we observed was a reduction of CD40^{+} macrophages at PBW 3 (Supplementary Figure 2D). Using FlowSOM analysis of the FCM data, we identified macrophage subtypes with different expression patterns: CD163^{-} macrophages (cluster 1), CD163^{+} macrophages with a low or moderate expression of CD40, CD80 and CD206 (cluster 2) and CD163^{+} macrophages with a moderate to high expression of CD40, CD80 and CD206 (cluster 3)

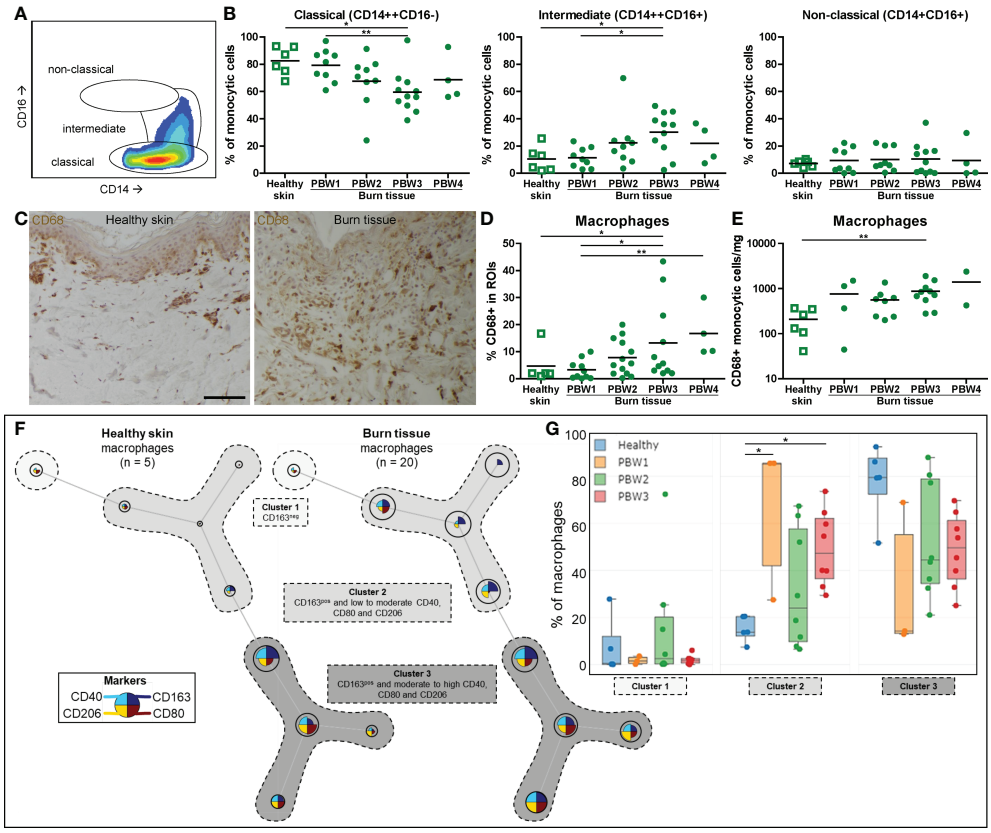


FIGURE 3
Local macrophage response to burn injury. **(A)** Flow cytometry gating strategy for detection of differentiation stages of monocytic cells (classical, intermediate or non-classical, as based on CD14 and CD16). **(B)** Flow cytometry-based quantification of percentage of monocytic cells within classical, intermediate, non-classical gates. **(C)** CD68 immunohistochemical DAB staining of a representative section of healthy skin and burn tissue (from 15 days post burn) (black scale bar = 100 µm). **(D)** CD68^{+} area of tissue sections. **(E)** Flow cytometry-based quantification of absolute number of macrophages (CD68^{+} monocytic cells) per mg tissue. **(F)** Unsupervised clustering of macrophages (CD68^{+} monocytic cells) in healthy skin and burn tissue, 3 clusters are highlighted. Node size represents relative size of population and node diagram shows expression level of markers. **(G)** Percentage of macrophages (CD68^{+} monocytic cells) within each cluster. Error bars in G show boxplot, p values were calculated using Mann-Whitney U statistical tests, significant differences are indicated by black asterisks: *p < 0.05; **p < 0.01.

(Figure 3F). A significant increase in macrophages in cluster 2 was observed in burn tissue at PBW 1 and 3 (Figure 3G). Overall, this analysis demonstrated that burn injury increased the number of macrophages and changed their composition.

Burn injury causes shifts in the lymphocyte composition and increases total T cells at PBW 2-3

T cell ($CD3^+$ lymphocyte) numbers rose significantly at PBW 2-3 (Figure 4A), in line with the total lymphocyte increase (Figure 1D). A shift towards more $CD4^+$ T cells was detected in burn tissue compared to healthy skin and were highest in burn tissue from PBW 3 as the CD4/CD8 T cell ratio ($CD3^+CD4^+/CD3^+CD4^-$ ratio) was higher in burn tissue than in healthy skin (Figure 4B and Supplementary Figure 2E). An increase in the proportion of γδ T cells ($CD3^+CD4^-\gamma\delta TCR^+$ lymphocytes) was found only at PBW 1 (Figure 4C), indicating a fast response of γδ T cells after burn injury. The absolute number of γδ T cells steadily increased over time after burn injury (Supplementary Figure 2F). The shift towards a higher abundance of γδ T cells at PBW 1 was confirmed by mapping flow cytometry data of T cells using FlowSOM (clusters 3 and 4; Figures 4D, E) and shows that the majority of the γδ T cells was $CD25^+$, which is a prominent marker for cellular activation (24). At PBW 1 there was a relative decrease of T cells with a regulatory phenotype ($CD25^+CD127^-$; cluster 1). We did not observe considerable alterations in the cluster containing $CD3^+CD4^-$ T cells (cluster 5).

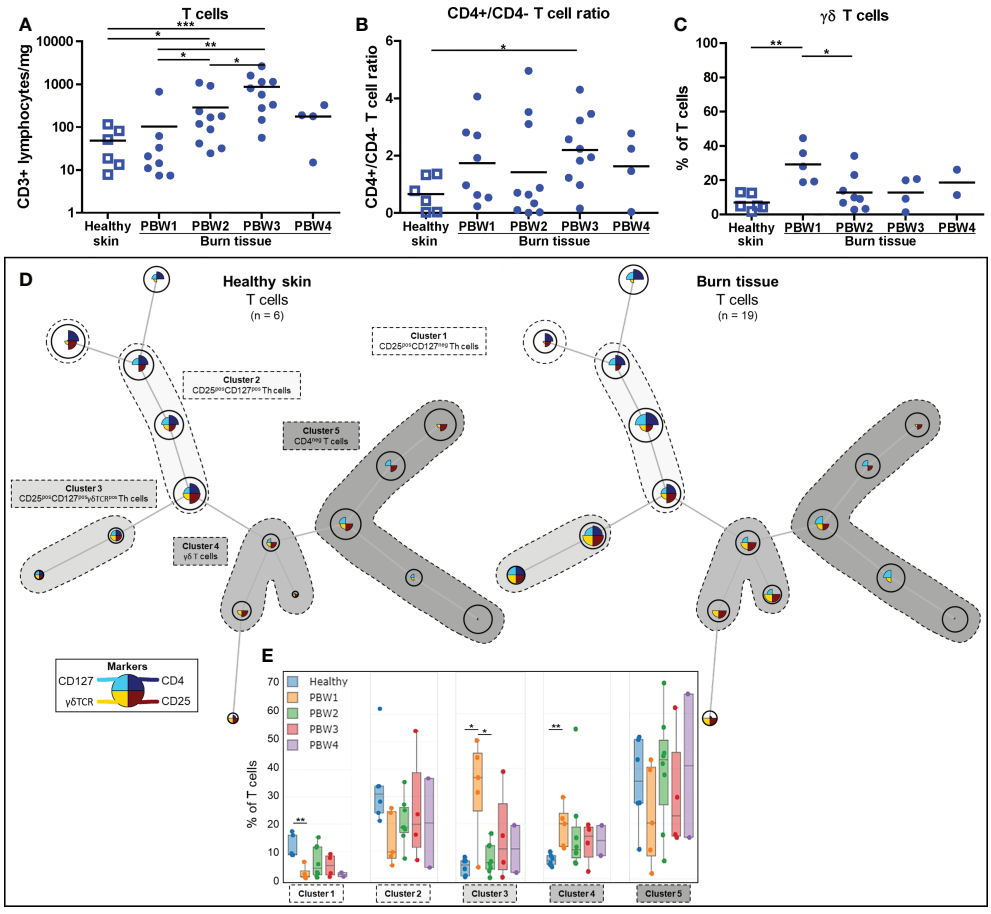


FIGURE 4
Local T cell response to burn injury. Flow cytometry-based quantification of: **(A)** Absolute number of T cells ($CD3^+$ lymphocytes) per mg tissue; **(B)** $CD4^+/CD4^-$ T cell ($CD3^+$ lymphocytes) ratio in tissue; **(C)** Percentage of T cells ($CD3^+$ lymphocytes) that are γδ T cells ($\gamma\delta TCR^+CD4^-$ T cells). **(D)** Unsupervised clustering of T cells ($CD3^+$ lymphocytes) in healthy skin and burn tissue, 5 clusters are highlighted. Node size represents relative size of population and node diagram shows expression level of markers. **(E)** Percentage of T cells within each cluster. Error bars in E show boxplot, p values were calculated using Mann-Whitney U statistical tests, significant differences are indicated by black asterisks: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

Absolute number of NK and B cells increase after burn injury

FCM analysis showed that the absolute number of NK cells ($CD56^+$ lymphocytes) was higher in burn tissue from PBW 2-3 (Figure 5A). Relative to total leukocyte numbers, NK cells were significantly reduced in burn tissue from PBW 1 and normalized afterwards (Supplementary Figure 2F). In both healthy skin and burn tissue, the majority of the NK cells was $CD16^-$ (Figure 5B), which is opposed to the NK cell composition in peripheral blood where approximately 90% of the NK cells are $CD16^+$ (25). Differences in CD16 expression of the NK cells were not observed between healthy skin and burn tissue or between time points. The absolute number of B cells ($CD19^+$ lymphocytes) were higher in burn tissue from PBW 3 (Figure 5C), while the proportion of B cells within the leukocyte population in burn tissue was similar to that of healthy skin (Supplementary Figure 2G). We identified 4 clusters using FlowSOM analysis of the FCM data: $CD56^+CD16^+$ NK cells, $CD56^+CD16^-$ NK cells, $CD9^+CD56^+$ B cells and $CD9^{low}CD56^-$ B cells (Figure 5D). Clustering analysis showed a clear shift towards more $CD9^{low}CD56^-$ B cells in burn tissue but no significant differences were detected over time (Figure 5E).

Immune cell infiltration coincides with high levels of cytokines, chemokines and growth factors

The concentrations of 37 soluble immune factors were determined in homogenates of burn tissue using Luminex immunoassay (raw data is presented in Supplementary Figure 3). Figure 6 shows an overview of these results using volcano plots and heatmaps at 4 time intervals after burn injury. In burn tissue there was an extremely high expression of IL-6,

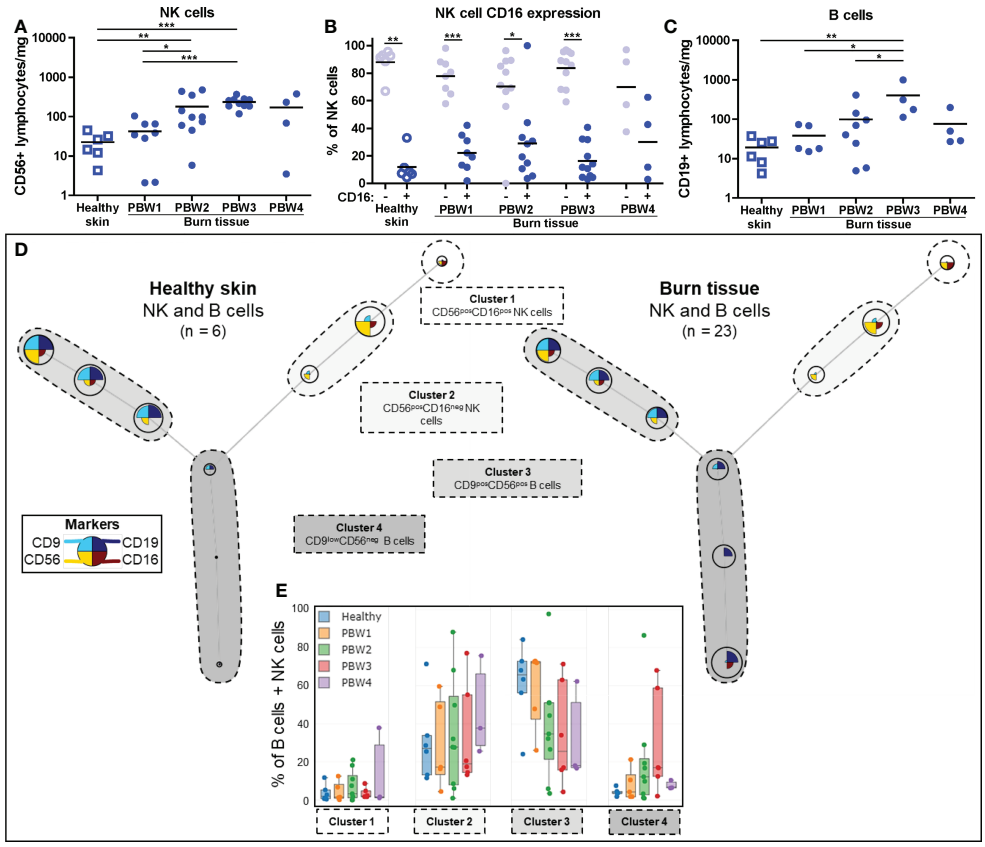


FIGURE 5
Local NK and B cell response to burn injury. Flow cytometry-based quantification of: **(A)** Absolute number of NK cells ($CD56^+$ lymphocytes) per mg tissue; **(B)** Percentage of NK cells that are $CD16^-$ and $CD16^+$; **(C)** Absolute number of B cells ($CD19^+$ lymphocytes) per mg tissue. **(D)** Unsupervised clustering of NK and B cells in healthy skin and burn tissue, 4 clusters are highlighted. Node size represents relative size of population and node diagram shows expression level of markers. **(E)** Percentage of NK or B cells within each cluster. Error bars in E show boxplot, p values were calculated using Mann-Whitney U statistical tests, significant differences are indicated by black asterisks: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

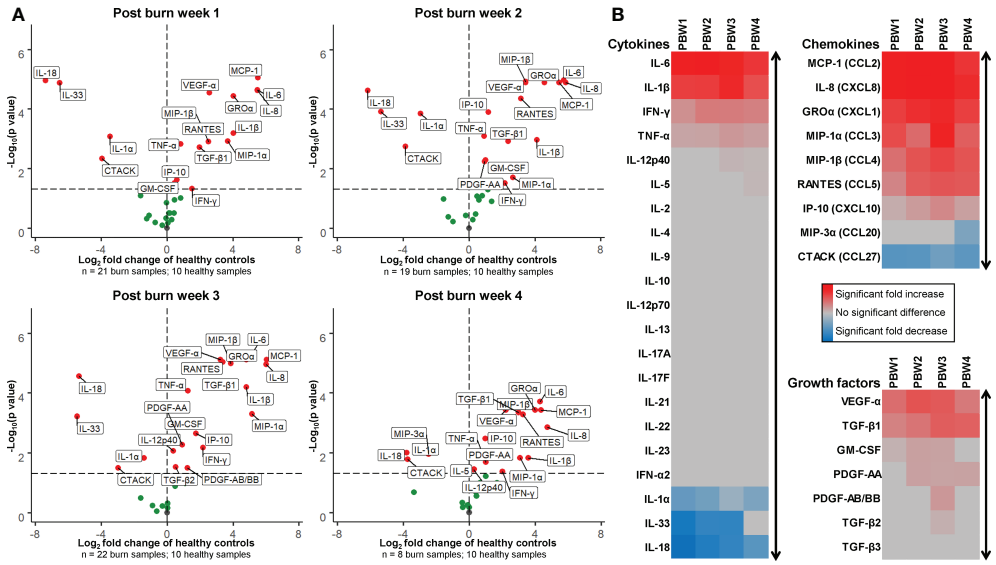


FIGURE 6
Expression of cytokines, chemokines and growth factors in burn tissue. **(A)** Volcano plot visualization of the expression of soluble factors in burn tissue from 1, 2, 3 and 4 weeks post burn injury. Dots represent soluble factors in burn tissue as Log_2 fold change as found in healthy skin controls. Factors with a statistically significantly different expression ($p < 0.05$) are labeled (values above the black striped line). **(B)** Heatmap visualization of fold increase/decrease of soluble factors in burn tissue compared to healthy skin, categorized by cytokines, chemokines and growth factors. P values were calculated using Mann-Whitney U statistical tests.

IL-1β, IFN-γ and TNF-α compared to healthy skin. The levels of these factors were persistently high, but for IL-6 and IFN- γ the levels declined at the later time intervals. Interestingly, increased levels of IL-12p40 and IL-5 were found only late after burn injury (PBW 3-4). As compared to healthy skin, a decrease was found for IL-1 family members IL-1α, IL-33 and IL-18. This is opposed to the level of IL-1β, which is also an IL-1 cytokine. The levels of IL-1α, IL-33 and IL-18 somewhat normalized at PBW 4 to the levels found in healthy skin. Chemokines MCP-1, IL-8, GROα, MIP-1α, MIP-1β, RANTES, IP-10 in burn tissue were increased at all analyzed time intervals, while the levels of T cell attracting chemokines CTACK and MIP-3α were decreased at PBW 1-4 and PBW 4, respectively. Among the growth factors, an increase in VEGF-A and TGF-β1 levels was found at PBW 1-4. From the growth factors, the level of GM-CSF was increased found at PBW 1-3, PDGF-AA at PBW 2-4 and PDGF-AB/BB and TGF-β2 only at PBW 3.

Discussion

Next to being a protective, physical barrier, the skin carries out immune surveillance to ensure early and effective defense mechanisms against external threats. Besides fibroblasts and keratinocytes, healthy skin is inhabited mainly by lymphocytes and antigen presenting cells that survey the skin and react to foreign structures and danger signals (26). Here, we aimed to provide detailed insight in the cellular and soluble immune response in burn injured skin during the first four weeks after injury. In this study, we showed that after burn injury, there is a fast, extensive and long-lasting increase in innate immune cells that is present even in burn tissue debrided 3 to 4 weeks after injury. Lymphocytes also rise in numbers, but mainly at PBW 2-4. In addition, the cytokine composition in these burn tissue samples is highly pro-inflammatory and likely continues the attraction and activation of immune cells. Excessive pro-inflammatory immune responses and a lack or delay of anti-inflammatory responses could complicate wound healing and patient recovery. Limitations of this study that should be addressed are minor differences in treatment between patients such as medication and timing of surgery that could have influenced the inflammatory response. In addition, the broad range in subject age, burn cause and TBSA could have increased variation in the responses.

In tissue samples from PBW 1, the proportion of γδ T cells was increased, indicating that γδ T cells could play a role during the early phase of burn-induced response. γδ T cells possess a unique T cell receptor and can, unlike αβ T cells, interact with antigens directly (27). They execute immune surveilling functions and react to damaged cell structures by producing cytokines and chemokines to recruit immune cells (28). Mouse studies have shown that γδ T cells regulate the infiltration of innate immune cells shortly after trauma (29, 30). Our data suggests that next to keratinocytes, fibroblasts, mast cells and

platelets (31), γδ T cells could be important inducers of the inflammatory response in humans as well. Within the same timeframe (PBW 1), IL-1β, IL-6, IL-8 (CXCL8), MCP-1 (CCL2), and GRO-α (CXCL1) levels were highly augmented. Others have demonstrated that these cytokines are also elevated in burn wound exudate (32). These factors are known enhancers of the inflammatory response and attract neutrophils and monocytes/macrophages to wound site (33). On the contrary, levels of IL-1α, IL-18 and IL-33 in burn tissue were reduced, especially during at PBW 1. These IL-1 cytokine family members are constitutively produced by keratinocytes to maintain the immune surveillance aspect of the skin (34). Reduction of these factors is presumably caused by extreme loss of keratinocytes due to destruction of the epidermal layer by thermal injury. In burn tissue from PBW 2-4, the levels of IL-1α, IL-18 and IL-33 were returning to the levels in healthy skin, which may be related to the presence of keratinocytes closing the defect. Levels of cytokines, as well as microRNAs (35), could be potential biomarkers to predict disease progression or recovery (36).

The rapid neutrophil response to burn injury is presumably caused by the persisting levels of neutrophil attractants, such as IL-8, MCP-1 and GRO-α. This can also be observed in the circulation of burn patients, where high levels of neutrophils were accompanied by high levels of IL-8 and MCP1, especially early after injury (8). Other studies have also shown that burn tissue contains large numbers of neutrophils in both human (37) and animals (14, 38). The vast majority of neutrophils that infiltrated the wound area were mature, whereas, in peripheral blood from severely burned patients high numbers of immature neutrophils were detected (8). This release of immature neutrophils may well be a compensatory response by the bone marrow (39). Nucleus flexibility and chemotactic activity increases with neutrophil age and could explain the presence of mainly mature neutrophils in burn tissue (40). If only mature neutrophils are able to enter the wound site, immature neutrophils would be trapped in the circulation until they reach maturity. As immature neutrophils are proposedly more active and less predictable in reacting to danger signals (41), they are likely to enhance systemic inflammation, thereby delaying recovery. In burn tissue, we found only a small number of immature neutrophils and only at PBW 1, which could have been released from the blood circulation by capillary leakage caused by the burn injury. Expression of CD11b and CD66b was increased on neutrophils isolated from burn tissue. This highlights the inflammatory state of the infiltrating neutrophils as CD11b and CD66b are important for neutrophil activation, adhesion and migration to inflamed tissue (42, 43). The surges of active neutrophils in the wound could lead to an overproduction of products such as elastase, MPO and ROS which can (further) damage surrounding tissues and organs (44, 45).

Blood monocytes are progenitors of both pro-inflammatory macrophages and wound healing macrophages. Although there is little evidence in this respect, it has been suggested that classical monocytes could be predisposed progenitors to pro-inflammatory macrophages (46), while intermediate and non-classical monocytes are biased progenitors to wound healing macrophages (47, 48). The initial monocyte population in burn tissue consisted mainly of classical monocytes. The relative decrease in classical monocytes in PBW 3 could indicate a relevant shift towards more wound healing macrophages, which is assumed to happen during wound healing (9). In burn tissue, the number of macrophages was increased this population showed a different composition of M1 and M2 markers. $CD163^+$ macrophages with low to moderate expression of CD40, CD80 and CD206 were more abundant in burn tissue. M1 macrophage differentiation factor GM-CSF was increased in burn tissue from PBW 1-3 and mediators that are known to be actively produced by M1 macrophages such as TNF-α, IFN-γ, IL-1β, IL-6, IL-8 and MCP-1 (CCL2) (9, 49), were all increased in these burn tissues. While typical M2 macrophage factors like IL-4, IL-10, IL-13 were unaffected, the levels of TGF-β1 and VEGF-α, which are also described as M2 mediators (48), were increased in burn tissue. Altogether, the monocyte/macrophage composition and cytokine environment possibly supports the generation of macrophages with a pro-inflammatory phenotype. Timely transition towards more suppressive, regenerative macrophages is however essential for a healthy healing process, as these cells support fibroblasts in the formation of collagen and enhance re-epithelization (9, 48). Due to the active, continuing inflammation and the presence of danger signals from tissue damage, macrophage transition might be delayed or insufficient, although more research is required to elucidate this.

Immunosuppression from the adaptive arm of the immune system is essential to create an environment in which fibroblasts and keratinocytes can repair the damaged skin (50). Here, we revealed that lymphocyte numbers, including T cells, NK cells and B cells, were increased at PBW 2-3, which is relatively late after injury (11). This coincided with a high levels of chemokines MIP-1α (CCL3), MIP-1 β (CCL4) and RANTES (CCL5), which are known to attract lymphocytes to injured skin (11). Particularly CCL3, CCL4 and CCL5 are involved in the activation of NK cells (51) and could lead to increased cytokine release by NK cells in burn tissue. Information on the response of NK cells and B cells in burn tissue is very limited at this moment. We here showed that after burn injury there is an increased number of NK cells and B cells in burn tissue, however, functional assays are needed in order to speculate about their behavior and involvement in the burn immune response. The levels of CCL3, CCL4, CCL5, IFN-γ, TNF-α and IP-10 (CXCL10) were associated with the number of T cells at

PBW 3. IP-10 promotes chemotaxis and inflammation and is likely induced by IFN-γ. Peters et al. previously described an interplay of keratinocytes and T cells and showed that IP-10 is actively produced by keratinocytes in co-culture, even with relatively low numbers of keratinocytes (52). This interplay is presumably also active during burn wound healing by residual, surrounding or re-epithelializing keratinocytes. Cytokines with anti-inflammatory properties such as IL-4, IL-10 and IL-13 were not detected in these burn tissue samples. Altogether, the soluble factors in burn tissue are likely to support Th1 response, resulting in more attraction of leukocytes to the wound site, while control or suppression of inflammation appears to be limited.

In this study, we showed that after burn injury, the numbers of immune cells were persistently elevated, while during normal wound healing neutrophils disappear within days and lymphocytes counts start to increase in the first week (10, 53, 54). Burn injury often leads to a prolonged hyperinflammatory state (3, 55) and treatment of burn wounds is therefore a difficult and time-consuming process. Damage to the skin is a trigger for the immune system to recruit immune cells en masse and replenish these immune cells in the blood from the bone marrow. Ancillary damage and chemokine production by immune cells and stressed skin cells will trigger the immune system to react, thereby creating a vicious circle of prolonged inflammation in both the skin and in the blood. Therapy is often empiric due to the large diversity among patients and their injuries, e.g. burn type, size, depth and location. In the present study, there was no indication that burn size or burn cause (water versus flame) affected cellular or soluble inflammatory markers (data not shown). Excessive and persistent inflammation is also among the causes of long-term complications such as the formation of hypertrophic scars (7). On top of that, there is a risk of contracting an infection and the activity of the immune system is unpredictable. In clinical practice, patients with burns larger than 15% TBSA are hypermetabolic and often develop SIRS or organ insufficiency. Hence debridement of burn tissue is important to reduce inflammation and promote wound healing while also preventing further tissue necrosis and cellulitis. Possibly, early debridement of burn tissue (noted as post-burn days 2 through 12) or impediment of pro-inflammatory cytokines such as IL-6 might remove inflammatory triggers at an early stage and avoid secondary damage (56, 57). Resolution of excessive inflammation using immune suppressants could increase the patients' recovery rate, but might increase the risk for infection. Moreover, it can be very difficult to discriminate burn-induced SIRS from sepsis. Our analysis of the local immune reactions to burn injury aids in improving our understanding of burn-induced inflammation. This knowledge is needed to design more sophisticated and effective ways to diagnose and treat immune dysfunction and hyperactive inflammation. Immune modulating treatment targeting the disturbed immune processes will improve patients' overall health recovery time and scar quality.

In conclusion, through the characterization of immune cell subsets isolated from human burn tissue we demonstrated that burn injury induced a local persistent surge of pro-inflammatory immune cells and cytokines, while immunosuppression appeared to be limited. These burn-induced immune reactions might be key factors that extent the inflammation phase and thereby obstruct the wound healing process in burn injury.

Data availability statement

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

Ethics statement

Tissue samples were obtained from planned surgeries that were part of routine patient therapy. Data and material was only used after consent from subjects (or legal representatives) using the opt-out procedure of the Red Cross Hospital in Beverwijk. This procedure is in accordance with the national guidelines (https://www.coreon.org/) and institutional guidelines of the Red Cross Hospital in Beverwijk in the Netherlands. No animals were used in these experiments.

Author contributions

Conceptualization: PM, MV, IJ, BB, and HK. Methodology: PM, MV, IJ, BB, HK, and AP. Investigation: PM, MV, and EF. Resources: MS, AP, and PVZ. Formal analysis: PM, MV, BB, and HK. Visualization: PM and MV. Supervision: IJ, BB, and HK. Writing – original draft: PM. Writing – review & editing: MV, EF, MS, AP, PVZ, IJ, BB, and HK. All authors have read and approved the submission of this manuscript.

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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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Supplementary material

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SUPPLEMENTARY FIGURE 1
Gating strategy of flow cytometric analysis. At the top of the plots the panel numbers are shown for which the gating was performed (P1, 2, 3 and 4).

SUPPLEMENTARY FIGURE 2
Cell counts in burn tissue. Flow cytometry-based quantification of: **(A)** Percentage of leukocytes that stained negative for 7-AAD or propidium iodide (viable cells); **(B)** Percentage of isolated cells that is CD45- (fibroblasts, keratinocytes, endothelial cells, others); **(C)** Number of eosinophils ($CD9^+CD16^-$ granulocytes) per mg tissue; **(D)** Percentage of macrophages that is positive for CD40, CD80, CD163 or CD206; **(E)** Percentage of T cells that are $CD4^-$ and $CD4^+$. **(F)** Number of γδ T cells ($γδTCR^+$) per mg tissue; **(G)** Percentage of NK cells ($CD56^+$ lymphocytes) within leukocyte population; **(H)** Percentage of B cells ($CD19^+$ lymphocytes) within leukocyte population. P values were calculated using Mann-Whitney U statistical tests, significant differences are indicated by black asterisks: *p < 0.05; **p < 0.01; ***p < 0.001.

SUPPLEMENTARY FIGURE 3
Concentrations of soluble factors in burn tissue. Healthy skin was used as controls. Black lines show mean values and the black striped line represents the lowest limit of detection. P values were calculated using Mann-Whitney U statistical tests, significant differences are indicated by black asterisks: *p < 0.05; **p < 0.01; ***p < 0.001.

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Over-expression of CRTH2 indicates eosinophilic inflammation and poor prognosis in recurrent nasal polyps

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Background: Chronic rhinosinusitis with nasal polyps (CRSwNP) is often characterized by recurrent nasal polyp (NP) growth following surgical removal, but the mechanisms are still not clear. This study aimed to investigate the expression of chemoattractant receptor-homologous molecule expressed on Th2 cells (CRTH2) receptor on NP and the role it plays in eosinophil inflammation and polyp recurrence.

Methods: Forty-one CRSwNPs patients and seventeen controls were enrolled in this study. mRNA was extracted from nasal tissues and evaluated for expression of CRTH2. Immunofluorescence staining was performed to confirm the distribution and expression of CRTH2 protein. CRTH2 expression on peripheral blood eosinophils was quantified by flow cytometry. The eosinophil count and clinical implications were also evaluated and their correlations with CRTH2 expression were analyzed.

Results: Nasal polyps displayed increased expression of CRTH2 in mRNA level compared with control samples, with the highest expression observed in recurrent NP. Immunofluorescence confirmed over-expression of CRTH2 in recurrent NP and this was independent of the concurrent presence of asthma. CRTH2 expression was positively correlated with tissue eosinophil number (Spearman's ρ=0.69, P<0.001) and the postoperative sino-nasal outcome test-22 (SNOT-22) score (Spearman's ρ=0.67, P<0.001). Receiver operating characteristic (ROC) curves revealed CRTH2 was more predictive for NP recurrence compared to either eosinophil number and concomitant asthma, with an area under the ROC curve of 0.9107.

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Conclusion: The over-expression of CRTH2 in recurrent nasal polyps correlates with greater eosinophilic inflammation and poor prognosis which is independent of concomitant asthma.

KEYWORDS

chemoattractant receptor-homologous molecule expressed on Th2 cells (CRTh2), chronic rhinosinusitis with nasal polyps, eosinophils, prognosis, recurrent nasal polyp

Introduction

Chronic rhinosinusitis (CRS) is divided into CRS with NP (CRSwNP) and CRS without NP (CRSsNP) (1) by the presence or absence of nasal polyps. A common feature of CRSwNP is the frequent recurrence of nasal polyps following surgical removal, especially for eosinophilic nasal polyps (2, 3), for which current therapies often prove inadequate (1, 4). Although studies have demonstrated eosinophilia is associated with the recurrence of nasal polyps (5–8), elimination of polyp eosinophils did not decrease nasal polyp size and symptoms in a clinical trial (9), suggesting, beyond eosinophils, there are still other mechanisms responsible for NP recurrence. Therefore, further explorations of the factors contributing to eosinophilic inflammation and nasal polyp recurrence are important for the treatment of CRSwNP.

Prostaglandin D_2 (PGD_2) is generally considered to be released by mast cells (10, 11), type 2 helper T lymphocytes (Th2) (12), macrophages (13), and eosinophils (14). It contributes to chemotaxis and activation of Th2 cells, eosinophils, basophils, and group 2 innate lymphoid cells (ILC2) through its receptor chemoattractant receptor–homologous molecule expressed on Th2 lymphocytes (CRTH2/DP2), and thus plays an important pro-inflammatory role in asthma and allergic diseases. Studies have shown that CRTH2 is up-regulated in patients with asthma and allergic rhinitis (15, 16), and CRTH2 receptor antagonists can reduce the antigen-induced increase in nasal airway resistance and local eosinophil infiltration in sensitized mice (17). In clinical trials, CRTH2 antagonists have been reported to be effective in the treatment of asthma and allergic diseases (18–20), especially in those with more eosinophils, but recent studies with higher level of evidence did not show a statistically significant improvement in the treatment of asthma (21–23), thus the clinical benefits of CRTH2 antagonists remained to be confirmed. However, these latter studies did not specifically recruit subjects likely to display high levels of CRTH2. Given the heterogenous responses to CRTH2 antagonists, there is a need to identify the most responsive disease endotype likely to respond to CRTH2 antagonism.

At present, the expression and the role that CRTH2 plays in CRSwNP is still controversial (24, 25), especially in those with recurrent nasal polyps, has not been reported. Our previous study (14) demonstrated over-expression of PGD_2 and hematopoietic prostaglandin D_2 synthase (hPGDS) in eosinophils of patients with aspirin-exacerbated respiratory disease (AERD), a condition that is characterized by recurrent nasal polyps (rNP), but the expression of CRTH2 receptors was not examined. Here, we further hypothesized that CRTH2 would be highly expressed in patients with rNP, which would thereby enhance the eosinophilic inflammation and contribute to the poor prognosis of CRSwNP. The current studies aimed to (i) investigate whether CRTH2 is highly expressed in patients with rNP and examine its distribution, (ii) analyze the association of CRTH2 expression with eosinophilic inflammation and prognosis of CRSwNP, and (iii) further explore the clinical implications of CRTH2.

Abbreviations: CRSwNP, chronic rhinosinusitis with nasal polyps; NP, nasal polyp; CRTH2, chemoattractant receptor-homologous molecule expressed on Th2 cells; ROC, receiver operating characteristic curves; CRS, chronic rhinosinusitis; PGD_2, prostaglandin D2; Th2, type 2 helper T lymphocytes; ILC2, group 2 innate lymphoid cells; hPGDS, hematopoietic prostaglandin D2 synthase; AERD, aspirin-exacerbated respiratory disease; rNP, recurrent nasal polyps; SNOT-22, the sino-nasal outcome test-22; HE, hematoxylin and eosin; hpfs, high-power fields; EDTA, ethylene diamine tetraacetic acid; ELISA, enzyme-linked immunoassay; AUC, area under the curve.

Methods

Patient recruitment

This study was case-control designed. Subjects who satisfied the diagnostic criteria for CRSwNP established by European Position Paper on Rhinosinusitis and Nasal Polyps (EPOS2012) (26) and subjects with deviated nasal septum were recruited from patients referred to the Department of Otolaryngology, Qilu hospital of Shandong University for endoscopic nasal surgery from January 2018 to October 2020. Excluded were subjects with fungal sinusitis, cystic fibrosis, immunodeficiency diseases, or tumors. Patients treated with a systemic glucocorticoid within 4 weeks prior to recruitment were also excluded as glucocorticosteroids may theoretically influence mediator release. Finally, 41 CRSwNPs and 17 patients with deviated nasal septum (control group) were

included. The postoperative therapeutic regimens included daily nasal saline irrigation, mometasone furoate spray 200 μg once daily for 12 weeks and endoscopic debridement. All of the CRSwNP patients were followed postoperatively for more than 12 months *via* endoscopy. For the patients who were found to have recurrent nasal polyps during follow-up, postoperative methylprednisolone 24 mg daily × 14 days was applied. This study was approved by The Medical Ethics Committee of Qilu Hospital of Shandong University and all patients provided written informed consent before enrollment.

The non-recurrent nasal polyp group (Non-rNP) was defined as patients whose NP did not recur during the subsequent 12 months postoperatively. The CRSwNP subjects whose NP recurred according to CRSwNP diagnostic criteria (26) within the 12 months that followed after surgery were classified as recurrent nasal polyp group (rNP) (27). Concomitant asthma was diagnosed according to the Global Initiative for Asthma (GINA) 2014 (28) criteria and confirmed by a respiratory specialist in our hospital.

Clinical data and sample collection

Nasal polyps of patients with CRSwNP and middle turbinate mucosa of patients with deviated nasal septum were collected intraoperatively. All the tissues were divided into two portions, and one was fixed in a 10% neutral formaldehyde solution to embed in paraffin for immunohistochemical analysis, while the other was immediately snap-frozen in liquid nitrogen and stored at -80°C until processed. Due to the size of tissue samples, not all experiments were done on all participants. Blood samples were obtained to determine the circulating absolute eosinophil count before surgery. Each patient underwent a high-resolution CT scan of the paranasal sinuses and scored preoperatively with the Lund-Mackay scoring system (range: 0–24) (29) by an experienced physician. The SNOT-22 Questionnaire (30, 31) were assessed preoperatively and 12 months postoperatively.

RNA extraction and qRT-PCR

Quantitative real-time PCR was used to quantify mRNA levels of hPGDS and CRTH2. Total RNA was extracted using Trizol Reagent (Sigma-Aldrich Co., St. Louis, MO, USA) from nasal tissues according to the manufacturer's protocols. Concentration and RNA quality were determined using the Nanodrop 2000 Spectrophotometer (Thermo Fischer Scientific, USA). Reverse transcription was performed with 1μg of RNA in a 20 μl reaction using TaqMan Reverse Transcription kit (Vazyme Biotech Co., Ltd, China). Reactions went through 15 min at 50°C, 5 min at 85°C in a Bio-Rad thermocycler. PCR reactions contained 15 ng of cDNA (total RNA equivalent) in 5ul HiScript III RT SuperMix for qPCR and primers. The assay was performed using the ABI 7900HT Fast Real-time PCR system (Applied Biosystems, USA). The primers for CRTH2 were as follows: sense, 5'-TGCAAACTGCACTCCTCCAT-3', antisense, 5'-AACACGAAATAGGGCACCGT-3', primers for hPGDS were: sense, 5'-GGGCAGAGAAAAACAAGATGT-3', antisense, 5'-CCCCCCTAAATATGTGTCCAAG-3'. Data were analyzed as the change in cycle threshold (CT) of each cytokine transcript in comparison with β-actin. β-actin primers 5'-GAAGAGCTACGAGCTGCCTGA-3' and 5' -CAGACAGCA CTGTGTTGGCG-3' were purchased (Biosune Biotechnology, Shanghai, China).

Hematoxylin-eosin staining

Nasal tissues were fixed in 4% paraformaldehyde, paraffin-embedded, and sectioned. Hematoxylin and eosin (HE) staining was performed and the eosinophil number was scored under 200× magnification in a blinded fashion. The eosinophils were counted with the final number being the average number of cells per 5 random high-power fields (hpfs).

CRTH2 immunofluorescence staining

Immunofluorescence staining was performed as described previously (32). The paraffin sections were deparaffinized and hydrated. Heat-induced antigen retrieval was performed by heating sections in a microwave oven for 20 minutes in citrate buffer. Slides were washed and blocked using 1% bovine serum albumin and 10% goat serum for 2 hours. Sections were incubated with rabbit anti-human CRTH2 antibody (Abcam, ab150632) at 1:150 dilution at 4°C overnight and then were rinsed with PBS (phosphate buffer saline) solution and incubated with secondary Alexa fluor 647 goat anti-rabbit antibody (1:200, Abcam, ab150079) for 1 hour at room temperature in dark. Nuclei were stained with 1000μg/ml DAPI (4', 6-diamidino-2-phenylindole) for 30 mins. The sections were sealed in antifade mounting medium. CRTH2 positive cells were scored in a blinded fashion. For the samples that were successfully stained, the slides were photographed using a confocal laser scan microscope LSM980 (Carl Zeiss, Germany) and the final number was expressed as the average number of cells per 5 random 200× hpfs.

Multiplexed immunofluorescence (mIF) assay was performed using the Opal 6-Plex Detection Kit (AKOYA #811001, USA). At room temperature, we incubated primary antibodies for major basic protein (MBP) (1:150; Boster, China), Siglec8 (1:30; Biolegend, USA), CRTH2 (1:150; Abcam, China), CD69 (1:150; Santa Cruz Biotechnology, China) for 2 hours. Following that, samples were incubated with the secondary antibody at 37°C for 10 minutes using the Opal ploymer anti-rabbit/mouse horseradish peroxidase (HRP). The Opal 6-Plex

Detection Kit was subsequently used to visualize the Tyramide Signal Amplification (TSA), which contains Opal 520, Opal 570, Opal 620, and Opal 690. Then the nuclei were stained with DAPI (1:100) for 10 minutes. Image acquisition was performed with TissueFAXS (TissueGnostics).

Flow cytometry

Peripheral blood was extracted from subjects in EDTA (ethylene diamine tetraacetic acid) tubes and then 150 µL was pipetted into respective polypropylene tubes. Negative controls only consisted of tested blood without the addition of diagnostic antibodies. ACK (Ammonium Chloride-Potassium) Lysis buffer was used to lyse red blood cells. Cells were resuspended in FACS (fluorescence-activated cell sorting) buffer at a concentration of 1 x 10^6 cells/ml and stained with 5 µL Zombie Aqua™ Fixable Viability (Biolegend, 423101), 5 µL FITC-conjugated anti-CD16 (BioLegend, 302006), and 5 µL APC-conjugated anti-CD294 (CRTH2) (Biolegend, 350109). Finally, the cells were harvested and resuspended in 500 µL PBS. Flow cytometry was performed including live/dead viability gate with CRTH2+ eosinophils identified as SSC^{high}, CD16- and CRTH2+ granulocytes. Isotype antibody controls were used to develop the gating and data analysis strategies. Flow cytometry was performed on BD FACSAria III. The data were analyzed with FlowJo.

ELISA for PGD_2

In brief, the nasal tissues were added to 1 mL of PBS per every 0.1 g of tissue and then homogenized on ice. After homogenization, the suspensions were centrifuged at 3,000 rpm for 10 minutes at 4°C, after which the supernatants were separated and stored at -80°C until analysis. Supernatants were assayed for the levels of PGD2 by using ELISA kits for PGD2 (Cayman Chemical, 512031) according to the manufacturer's directions (lower limit of detection, 55 pg/mL).

Statistical analyses

The data generated in this study were analyzed using GraphPad Prism 8 and SPSS version 26.0. Continuous data were represented as mean with standard deviation (SD) and were assessed for normality and equal variation. Chi-square test was applied for categorical variables to compare the demographic distribution and clinical variables among the different groups. One-way analysis of variance (ANOVA) was performed for comparisons of rNP, Non-rNP, and control data that passed the normality and equal variation tests, otherwise, Kruskal-Wallis test was used. The subsequent multiple comparisons for two groups were further adjusted using the Holm-Sidak's multiple comparisons test and Dunn's multiple comparisons test, respectively. Correlation analysis was performed using Spearman's correlation by comparing eosinophils and SNOT-22 as a function of CRTH2 positive cell number, considering the non-normality. The ROC curve was plotted to compare the predictive ability among different variables. A P value of <0.05 was considered statistically significant.

Results

Demographic and clinical characteristics

Demographic and clinical characteristics of subjects are shown in Table 1. There were no significant differences in age, sex, allergic rhinitis, hypertension, diabetes mellitus, smoking, and drinking among the three groups. The percentage of patients with asthma was significantly higher in rNP than those in control group (P = 0.006). Due to the size of tissue samples, we cannot perform every experiment on all the participants. The numbers of individuals that were enrolled in each experiment were shown in each dot Figures 1, 2, 4, 5, 7 and Supplement Figure 2.

Screening of hPGDS-PGD_2-CRTH2 pathway in nasal polyps

To explore the role that hPGDS-PGD_2-CRTH2 pathway plays in CRSwNP, especially in the recurrence of nasal polyps, we screened the expression of hPGDS-PGD_2-CRTH2 in nasal polyps defined by their tendency to recur post-surgery. No significant differences were observed among these groups in tissue hPGDS mRNA levels (Figure 1A) and PGD_2 levels of tissue homogenates measured by ELISA (Figure 1B). In contrast, CRTH2 mRNA level in rNP group was significantly higher compared with those in Non-rNP (Figure 1C, P = 0.04) and control groups (Figure 1C, P = 0.006). Taken together, these data suggest a prominent role of CRTH2 in the hPGDS-PGD_2-CRTH2 pathway of rNP.

Immunofluorescence staining of CRTH2 in nasal polyps

To detect the level of CRTH2 protein and define the cellular source, we performed immunofluorescence staining on nasal tissue samples. Representative images were shown in Figure 2, and the isotype control images were displayed in Supplement Figure 1. Essentially no CRTH2 staining was observed in control tissue (Figure 2A), moderate CRTH2 levels were detected in Non-rNP samples (Figure 2B), and the highest levels were found in rNP (Figure 2C). As shown in Figure 2D, most of these cells in rNP were supposed to be eosinophils by typical bilobed nuclei

TABLE 1 Characteristics of subjects enrolled in this study.

Characteristics	Recurrent nasal polyp .(n=26)	Non-recurrent nasal polyp (n=15)	Control group (n=17)	*P* value
Age, mean ± SD, y	41.7 ± 15.1	50.7 ± 9.6	37.8 ± 17.5	0.062
Female, No. (%)	8(30.7)	5(33.3)	6(35.2)	0.952
Asthma, No. (%)	11(42.3)	3(20.0)	0(0.0)	0.006
Allergic rhinitis, No. (%)	9(34.6)	2(13.3)	3(17.6)	0.234
Hypertension, No. (%)	6(23.0)	2(13.3)	4(23.5)	0.716
Diabetes mellitus, No. (%)	1(3.8)	1 (6.6)	0(0.0)	0.581
Smoking, No. (%)	6(23.0)	5(33.3)	2(11.7)	0.342
†Drinking, No. (%)	11(42.3)	7(46.6)	3(17.6)	0.160
Blood eosinophil count (10^9/L), mean ± SD	0.48 ± 0.07	0.24 ± 0.03	0.14 ± 0.26	<0.001
Lund-Mackay score, mean ± SD	16.6 ± 5.7	13.5 ± 5.6	–	0.118

SD, standard deviation; No, number.
†A regular drinker was defined as a person who drank alcoholic beverages at least once a month.

(yellow arrows). To further explore the expression of CRTH2 in nasal polyps, the number of CRTH2+ cells per hpf in immunofluorescence staining was determined. The rNP group presented with more CRTH2+ cells compared with Non-rNP (Figure 2E, $P = 0.03$) and control group (Figure 2E, $P < 0.001$). These results further support our mRNA data showing high expression of CRTH2 in rNP. In order to confirm the CRTH2 expression on eosinophils, Siglec8 and MBP were used as eosinophil markers in multiplexed immunofluorescence staining (Figure 3) on these tissues, which showed that most of the CRTH2 positive cells were co-stained with Siglec8 and MBP, especially in the rNP group. Overall, these data indicated the higher expression of CRTH2 in rNP and the close relationship between CRTH2 and eosinophils.

High CRTH2 expression in rNP is not driven by concomitant asthma

As more recurrent NP subjects had concomitant asthma (Table 1) and previous research had reported the higher expression of CRTH2 in asthma (33), to further address whether the CRTH2 difference between rNP and Non-rNP was driven by asthma, we analyzed the expression of CRTH2 in nasal polyps obtained from asthmatic and non-asthmatic subjects. However, no significant difference in CRTH2 expression was found between the asthmatic and non-asthmatic groups (Figure 4), demonstrating that higher CRTH2 expression may be an independent feature of rNP and that this is not being driven by the presence of asthma.

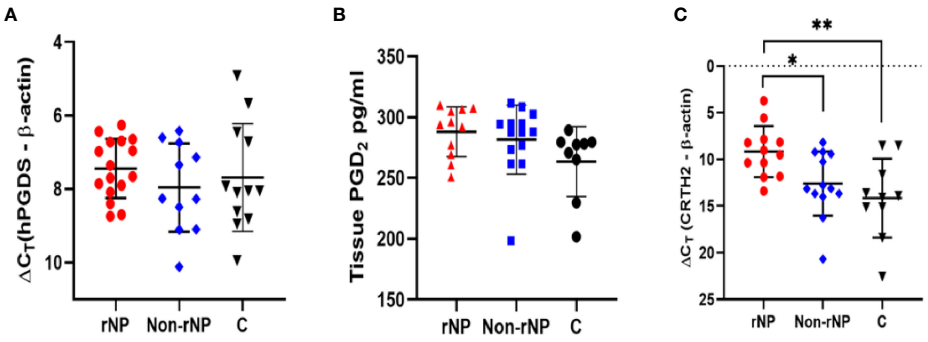

FIGURE 1
Screening of hPGDS-PGD_2-CRTH2 pathway in nasal polyps. Nasal polyp tissues from rNP and Non-rNP groups and middle turbinate mucosa of the control group were collected and detected by real-time quantitative PCR and ELISA. No significant difference was observed among the groups in tissue hPGDS mRNA level (rNP group: n = 16; Non-rNP group: n = 11; control group: n = 12) **(A)**. The PGD2 levels of tissue homogenates measured by ELISA did not show any significant difference among these groups either (rNP group: n = 11; Non-rNP group: n = 14; control group: n = 9) **(B)**. CRTH2 mRNA level in rNP was significantly higher compared with Non-rNP and control groups (rNP group: n = 12; Non-rNP group: n = 13; control group: n = 10) **(C)**. ΔCt (the Ct value of the target gene minus the Ct value of housekeeping gene β-actin) was used to compare the data among the groups. ΔCt value was inversely proportional to the relative level of cDNA contained in the sample. C, control group. *$P < 0.05$, ** $P < 0.01$.

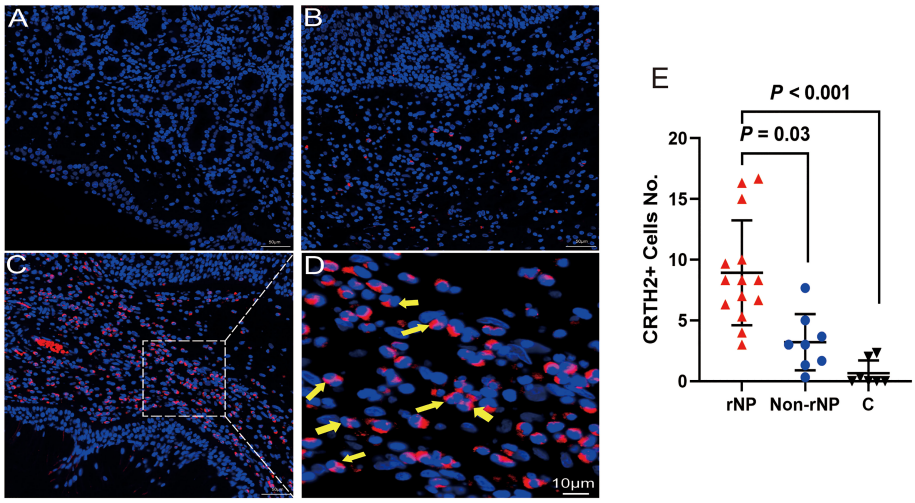

FIGURE 2
Immunofluorescence for CRTH2 (red color) in polyps from patients with NP and control tissue. **(A)** Control tissue. **(B)** Non-rNP tissue. **(C)** Tissue from a patient with rNP. **(D)** A close-up view of picture **(C)**, yellow arrows indicate eosinophils with bilobed nuclei. A dot plot showing positive CRTH2 cells/hpf for tissue samples from patients with rNP (n = 14) / Non-rNP (n = 8) / control subjects (n = 7) **(E)**. Images were generated as described in methods and analyzed by counting CRTH2+ cells with the exclusion of epithelial and glandular cells. C, control group.

Over-expression of CRTH2 is associated with eosinophilic inflammation in NP

As eosinophils were identified as being the most numerous cell expressing CRTH2 in nasal polyps, we further explored the association between CRTH2 and eosinophils in nasal polyps. It can be seen in Figure 5A that the circulating eosinophil count in the rNP group was significantly higher than that in the control group ($P < 0.001$). The counts of blood neutrophils, monocytes, and lymphocytes were also analyzed, but no significant differences were observed among these groups (Supplement Figure 2). To evaluate CRTH2 receptor expression on blood eosinophils, flow cytometry was performed and revealed a higher CRTH2 positive percentage in total eosinophils in patients with rNP compared with that in the control group (Figure 5B, $P = 0.03$), suggesting the blood eosinophils of rNP are more active with higher CRTH2 expression even on a per-cell basis. Gating strategy, isotype controls, and representative images are displayed in Supplement Figure 3.

To evaluate tissue eosinophils, HE staining was performed on nasal tissues. A prominent expression of eosinophils was observed in rNP, while almost no eosinophils were detected in control tissue (Figures 5C–F), with the tissue eosinophil number in the rNP and Non-rNP group significantly higher than that in the control group (Figure 5G, $P<0.001$ and $P=0.047$, respectively). Further analysis demonstrated tissue CRTH2+ expression positively correlated with tissue eosinophil number (Figure 5H, Spearman's $\rho=0.69$, $P < 0.001$). Additionally, to identify the characteristics of the CRTH2+ eosinophils, we also performed multiplexed immunofluorescence staining with CD69, a marker of eosinophil activation. Extensive co-stainings were observed among CD69, CRTH2, MBP, and Siglec8 in rNP tissues, suggesting an activated state of the CRTH2+ eosinophil in rNP, a phenomenon that was not shown in the control tissues (Figure 6). Taken together, these results indicate the expression of CRTH2 is closely associated with eosinophilic inflammation in NP patients.

Clinical implications of CRTH2 expression in patients with CRSwNP

The over-expression of CRTH2 in the rNP group and its close association with eosinophilic inflammation prompted us to query whether its expression affected the symptoms and prognosis of CRSwNP. Spearman's analysis revealed a positive correlation between the CRTH2 expression and the 12-month postoperative SNOT-22 score (Figure 7A, Spearman's $\rho=0.67$, $P<0.001$). The preoperative SNOT-22 score and Lund-Mackay score were also assessed, but no significant associations were detected between these data and CRTH2 expression (data not shown). Together these data suggest that higher CRTH2 expression may indicate a poor postoperative prognosis of NP.

To further address the factors predicting the recurrence of nasal polyps, ROC curves were performed. CRTH2 expression showed the highest accuracy as a predictor for rNP (Figure 7B, AUC=0.9107) compared with tissue eosinophil number (AUC=0.7354), concomitant asthma (AUC=0.6115), and blood

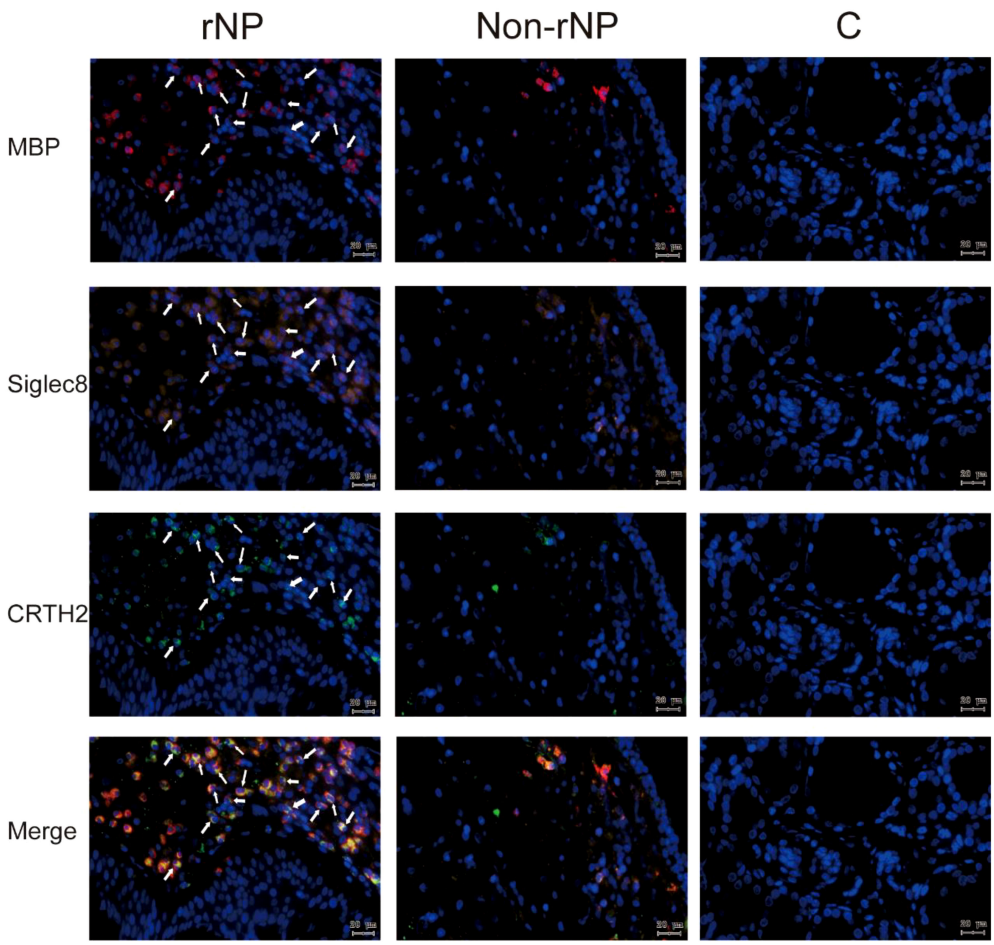

FIGURE 3
Multiplex immunofluorescence staining of nasal tissues for MBP (red), Siglec8 (orange), CRTH2 (green), and DAPI (blue) were performed. Representative fluorescence images of rNP group, Non-rNP group, and control group. White Arrows indicated eosinophils that expressed the corresponding markers. C, control group.

eosinophil number (AUC=0.7083). In this regard, when we set a cutoff point value of >5 for CRTH2 expression, the sensitivity and specificity for predicting rNP were 87.50% and 85.71%, respectively, an accuracy that could not be achieved by either eosinophil number or concomitant asthma.

Discussion

Eosinophilic CRSwNP is usually characterized by recurrent nasal polyp growth following surgical removal. Even in Asian populations, in which non-eosinophilic or neutrophilic polyps are the dominant type (34), a large number of tissue eosinophils can still be used as a reliable indicator for predicting nasal polyp recurrence (35). Consistent with these findings, we found that the circulating eosinophil number, while not that of other inflammatory cells (neutrophils, monocytes, and lymphocytes), was significantly higher in the rNP group than that in the control group (Figure 5A and Supplement Figure 2), and a more prominent difference in tissue eosinophils number was also observed between the rNP group and control group (Figure 5G). These observations support the important role that eosinophils play in rNP. The potential reason may be that eosinophils contribute to enhanced inflammation, tissue remodeling, a loss of matrix deposition, and an increase in tissue edema (36). Thus, understanding the factors that promote eosinophil migration and accumulation in the tissue is important for preventing nasal polyp recurrence.

PGD_2 can activate Th2 lymphocytes, eosinophils, basophils, and ILC2s, inducing cytokine production and enhancing chemotaxis through CRTH2 receptor engagement (37, 38), and thus play an important role in asthma and allergic diseases. The hPGDS-PGD_2-CRTH2 pathway was up-regulated in patients with severe, poorly controlled asthma

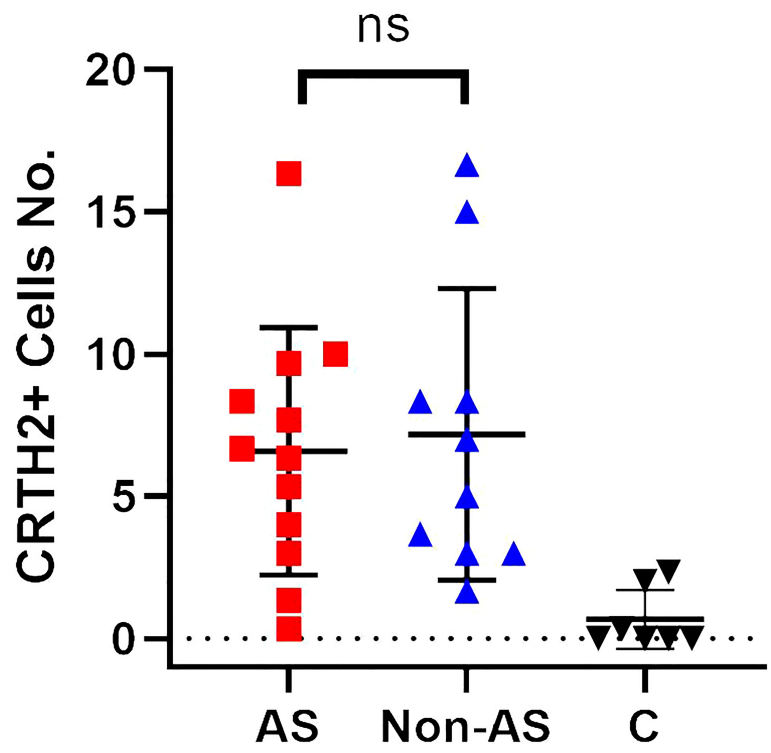

FIGURE 4
A dot plot showing positive CRTH2 cells/hpf for tissue samples from nasal polyps with concomitant asthma (AS) (n = 12) / nasal polyps without concomitant asthma (Non-AS) (n = 10) / control subjects (n = 7). ns, not significant.

(33). CRTH2 expression on leukocytes in allergic nasal mucosa was significantly up-regulated compared with those in nonallergic nasal mucosa (17). However, the exploration of CRTH2 in nasal polyps is still limited and debated. Yamamoto et al. demonstrated a down-regulation of CRTH2 in NP compared to uncinate process mucosae (24), but Nantel et al. reported CRTH2 was only detected in the nasal mucosa of subjects suffering from polyposis, while not in normal mucosa (25). Our previous research demonstrated over-expression of hPGDS-PGD_2 in eosinophils of AERD patients (14). Here, we further screened the hPGDS-PGD_2-CRTH2 pathway in nasal polyps and confirmed the over-expression of CRTH2 in NP, specifically in the setting of rNP. Interestingly, no significant differences were observed among rNP group, Non-rNP, and control groups in tissue hPGDS mRNA levels and PGD_2 levels of tissue homogenates in accordance with the findings of Nordström et al. which was based on nasal secretions (39). However, the detection by immunofluorescence staining confirmed higher CRTH2 expression in rNP compared to Non-rNP, suggesting a greater role of CRTH2 in the hPGDS-PGD_2-CRTH2 pathway driving rNP. Consistent with the study by Yamamoto and his colleagues (24), we also found that CRTH2 was selectively expressed in inflammatory cells, but they demonstrated a lower CRTH2 mRNA expression in NP, which is opposite to our research. This difference may be led by the different control tissues used in the two studies, as we chose middle turbinates of patients with deviated nasal septum as control, while Yamamoto et al. used uncinate process mucosae of CRS patients, which may be inflammatory mucosae. Further exploration is needed to elucidate these debates.

Of note, although more recurrent NP subjects had concomitant asthma (Table 1), CRTH2 expression was not greater in NPs derived from asthmatics, indicating that the over-expression of CRTH2 in nasal polyps is a specific feature of rNP independent of asthma status.

In asthma and allergic rhinitis, CRTH2 is essential for sustained eosinophilic inflammation (40). However, the relationship between CRTH2 and eosinophil inflammation in nasal polyps has not been reported. Here we demonstrated the close relationship between CRTH2 and eosinophilic inflammation in nasal polyps (Figure 5H). Moreover, our data also revealed the over-expression of CRTH2 on blood eosinophils of patients with rNP (Figure 5B), of interest, this over-expression in blood was not as prominent as that in tissue, arguably reflecting that the high-expressing CRTH2+ eosinophils had selectively migrated into tissue. In agreement with the findings of Miki-Hosokawa et al. the increased CD69 expression on activated eosinophils in rNP was also been found

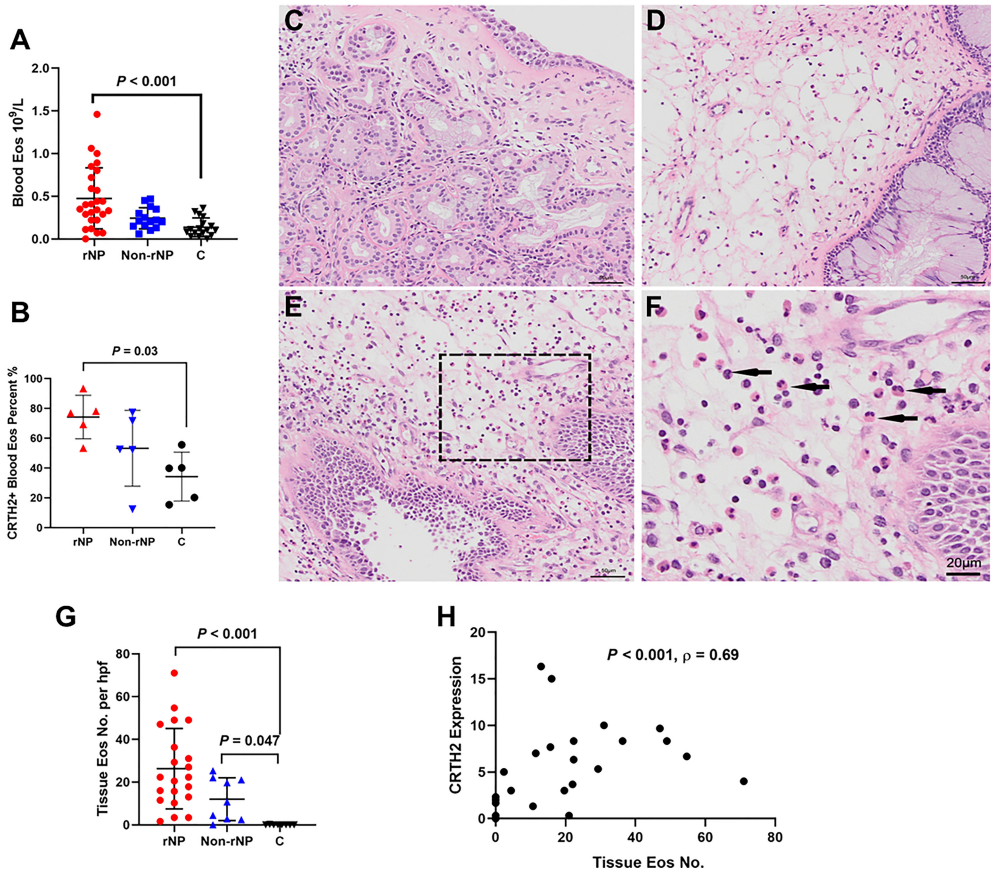

FIGURE 5
Over-expression of CRTH2 is associated with eosinophil inflammation in NP. **(A)** The blood eosinophil count in patients with rNP was significantly higher than that in the control group, while the difference between control and Non-rNP was not significant (rNP group: n = 26; Non-rNP group: n = 15; control group: n = 17). **(B)** Eosinophils of the rNP group presented higher CRTH2 expression than that of controls as shown by the higher percentage of CRTH2+ in blood eosinophils detected by flow cytometry (n = 5 per group). **(C)** HE staining of middle turbinate mucosa of the control group, **(D)** Non-rNP, **(E)** rNP, and **(F)** a closer view of picture **(E)**, with the black arrows indicating eosinophils. **(G)** Tissue eosinophils were further scored as mean number of eosinophils/200×hpf in HE stained sections. rNP (n = 21) and Non-rNP (n = 9) displayed significantly higher eosinophil numbers compared with controls (n = 8). **(H)** Correlation between eosinophils number and CRTH2+ expression detected by immunofluorescence in nasal tissues (n = 27). C, control group.

(41). Together these observations argue that the over-expression of CRTH2 may contribute to the migration and accumulation of eosinophils in rNP through enhancing the sensitivity of eosinophils to PGD_2, and thus contribute to this recurrence of nasal polyps. On the other hand, Th2 lymphocytes and ILC2s are also linked with tissue eosinophil accumulation and have a potential role in the activation and survival of eosinophils during the type 2 immune response (42). As such, we cannot exclude the impact of other inflammatory cells such as Th2 and ILC2 on NP recurrence. Further exploration of the relationship between CRTH2 and ILC2/Th2 and other cell types in rNP is warranted.

Eosinophilia and concomitant asthma were reported to be the risk factors of nasal polyps recurrence (5–8). In this research, we further demonstrated that up-regulated CRTH2 expression was linked with poor prognosis and, importantly, that CRTH2 expression was a better predictor for nasal polyp recurrence compared with either eosinophil expression or asthma diagnosis when analyzed by ROC. As eosinophils are not the only source of CRTH2-expressing cells, higher CRTH2 expression may also indicate higher numbers of activated Th2 cells, ILC2, and other inflammatory cells in rNP, which will aggravate inflammation, promote nasal polyp recurrence and contribute to the poor prognosis. As such, the expression of CRTH2 may be a good marker for all these inflammatory cells while not just eosinophils. Moreover, we also confirmed the significant difference between rNP and Non-rNP in tissue CRTH2 expression (Figure 2E) while not in tissue (Figure 5G) and blood eosinophils number (Figure 5A) in this study, indicating CRTH2 plays a more prominent role in NP recurrence than eosinophils. Collectively, these data may explain the better

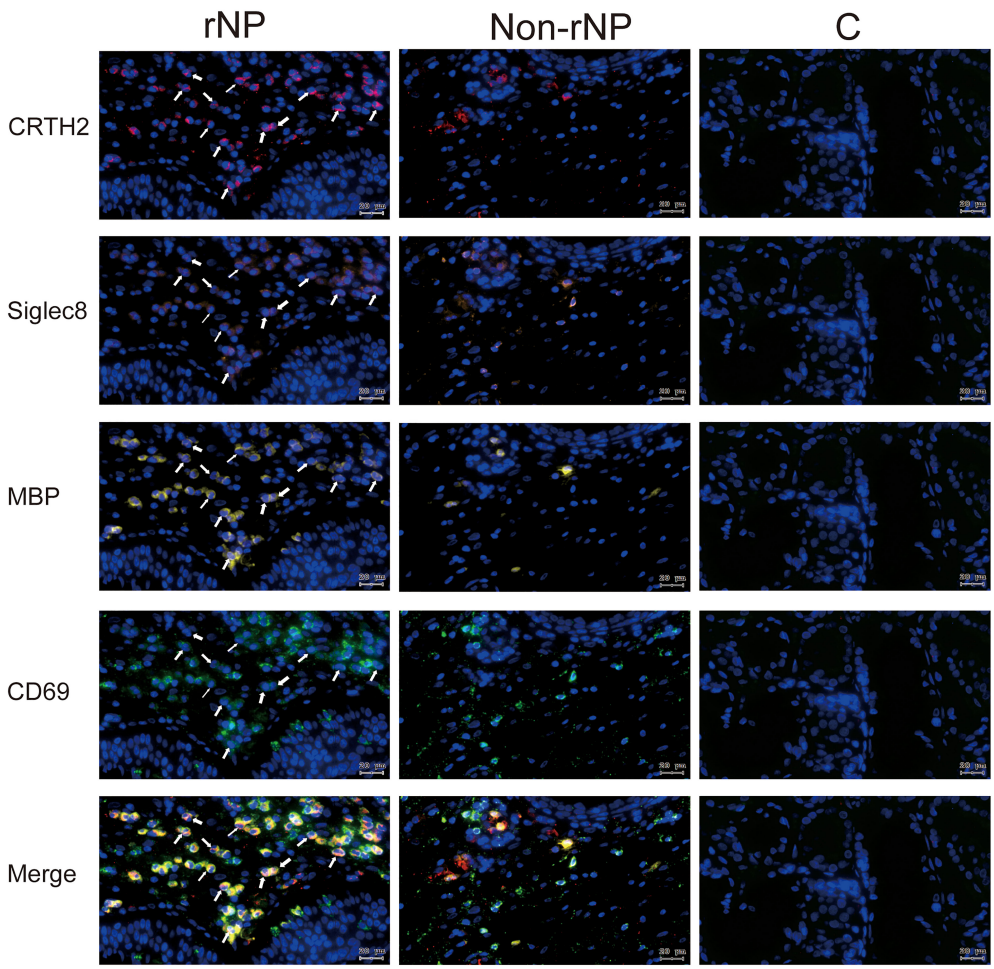

FIGURE 6
Representative tissue images stained using multiplex immunofluorescence staining for CRTH2 (red), Siglec8 (orange), MBP (yellow), CD69 (green), and DAPI (blue) in rNP group, Non-rNP group, and control group. White Arrows indicated eosinophils that expressed the corresponding markers. C, control group.

accuracy of CRTH2 expression in predicting NP recurrence and poor prognosis in comparison to that predicted by tissue eosinophils alone.

Taken together, these findings reminded us that whether CRTH2 antagonism could be a potential treatment option for eosinophilic CRSwNP and rNP, especially in those subjects displaying the highest level of this receptor. Several CRTH2 antagonists have been developed and tested in clinical trials of asthma and allergic diseases, but their clinical benefits still need to be determined (18, 19). CRTH2 antagonists Setipiprant (43), BI671800 (44), and OC000459 (45) were proved to be effective in treating allergic rhinitis symptoms. The CRTH2 antagonist fevipiprant safely improved asthma outcomes compared to placebo, but most of the differences did not reach the minimal clinically important difference (20–22, 46). To date, only one phase 3b study (NCT03681093) evaluated fevipiprant, as an add-on to nasal spray standard-of-care in reducing NP size in patients with nasal polyposis and concomitant asthma, but no prominent clinical benefits were observed, which may be explained by our study that NP with concomitant asthma did not present an over-expression of CRTH2 (47). Given the heterogenous responses to CRTH2 antagonists with some trials failing to produce clinically important differences (22), there is a need to identify the most responsive NP endotype likely to respond to CRTH2 antagonism. The up-regulated expression of CRTH2 in rNP and the significant positive correlation between CRTH2 and post-operative SNOT-22 in our results strongly suggest that recurrent CRSwNP may be one such subtype likely to benefit from CRTH2 antagonism which required to be further confirmed *in vivo* trails. Future studies that specifically recruit subjects displaying high levels of CRTH2, such as those with AERD or rNP, may be needed.

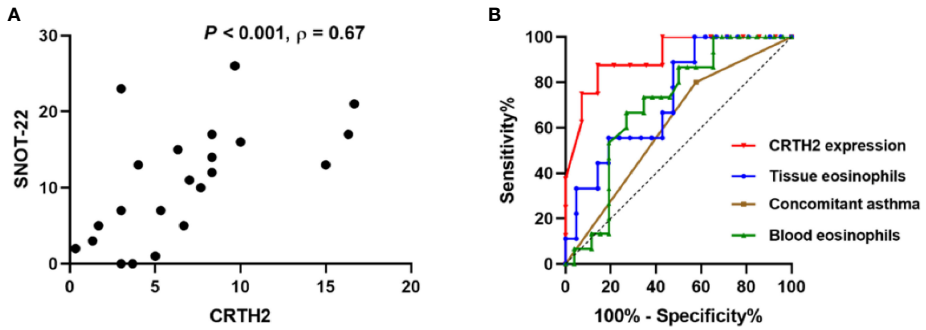

FIGURE 7
(A) Correlation between CRTH2 and SNOT-22 score 12-months postoperatively (n = 22). **(B)** Receiver operating characteristic curves of tissue CRTH2 expression (red line, AUC=0.9107, *P*=0.002, 95% CI: 0.7855 to 1.000), tissue eosinophil number (blue, AUC= 0.7354, *P*=0.044, 95% CI: 0.5497 to 0.9212), concomitant asthma (brown, AUC=0.6115, *P*=0.239, 95% CI: 0.4348 to 0.7883) and blood eosinophil number (green, AUC=0.7083, *P*=0.031, 95% CI: 0.5435 to 0.8642), respectively. AUC, area under the receiver operating characteristic curve.

There are several limitations to this study. The linkage of CRTH2 to the underlying biology is complicated in CRSwNP, as besides eosinophils, Th2, ILC2, and other inflammatory cells are also likely to be the source of CRTH2 over-expression and thus contribute to the recurrence of NP through this receptor, we cannot exclude the effects of these cells. *In vitro* experiments with purified eosinophils from rNP patients may be needed to determine the exact role that CRTH2 plays in eosinophil migration and accumulation. Second, PGD2 is also synthesized in necrotic tissues and cell lysates, therefore, the supernatants of tissue homogenates might not accurately reflect the physiological state of these tissues, further detection of PGD2 with nasal wash fluid or supernatants of the fresh tissues that were collected after washing the resting cells and tissue pieces may be needed. Finally, this study was performed just in the Chinese population, which may limit the external validity of this research, further studies with more samples and diverse races may be needed to validate the generalizability of this research.

Conclusion

This study demonstrated the over-expression of CRTH2 in rNP, a feature that is independent of concomitant asthma. Together with the findings that CRTH2 expression correlated with the extent of eosinophilic inflammation and postoperative SNOT-22 score, we may anticipate that in patients with the over-expressing phenotype, a CRTH2 antagonist may be a potential therapeutic option in patients with rNP that have proven to be unresponsive to standard therapy.

Data availability statement

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

Ethics statement

The studies involving human participants were reviewed and approved by The Medical Ethics Committee of Qilu Hospital of Shandong University. Written informed consent to participate in this study was provided by the participants or the participants' legal guardian/next of kin.

Author contributions

XF designed the study. WC, SH and XF drafted the manuscript. WC, SH, XX, PY, XF, CD and XL contributed to the enrollment of subjects and data collection. WC and SH performed the experiments. XY, SH and WC performed the statistical analysis. XF, XY, LB and ML contributed to interpretation of the results, reviewed and edited the manuscript. All authors contributed to the article and approved the submitted version.

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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.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.1046426/full#supplementary-material

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The research development of STAT3 in hepatic ischemia-reperfusion injury

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Ischemia-reperfusion injury (IRI) is a common complication of surgery, which can cause rapid deterioration of the liver function, increase the risk of graft rejection, and seriously affect the prognosis of patients. The signal transducer and activator of transcription 3 (STAT3) protein has been implicated in pathogenesis of IRI. STAT3 influences the mitochondria through multiple pathways and is also involved in apoptosis and other forms of programmed cell death. STAT3 is associated with Janus kinase (JAK), phosphoinositide-3 kinase (PI3K), and heme oxygenase-1 (HO-1) in liver IRI. The STAT3 pathway plays a dual role in IRI as it can also regulate lipid metabolism which may have potential for treating IRI fatty liver. In this review, we summarize research on the function of STAT3 in liver IRI to provide references for its application in the clinic.

KEYWORDS

signal transducers and activators of transcription3 (STAT3), ischemia-reperfusion injury (IRI), mitochondria, cell apoptosis, liver

Ischemia-reperfusion injury (IRI) is a common complication after liver surgery (such as surgery for liver cancer or liver transplantation). The interruption of the oxygen supply during ischemia causes hepatic sinusoidal stenosis as well as secondary microcirculation disorders (1, 2). Various factors such as tissue hypoxia, nutrient deficiencies, and metabolic disruption during ischemia can lead to hepatocyte injury. Inflammatory factors, apoptotic pathways, and reactive oxygen species (ROS), which are activated during reperfusion, can result in a rapid deterioration of the liver function, which also increases the risk of rejection and can adversely affect patient prognosis (3, 4). IRI is usually classified as warm IRI *in vivo* and cold IRI *in vitro*. Although both are primarily caused by hypoxia and the consumption of substrates caused by ischemia, the treatment methods differ due to the differences in the temperature and cell metabolic energy (5). Liver IRI is still a major problem in liver surgery, and no effective treatments are currently available. Signal transducer and activator of transcription (STAT) proteins are a class of transcription factors present in the cytoplasm, and mainly function to transmit signals from cell-surface receptors to the nucleus. The STAT family consists of seven distinct members, namely, STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B, and STAT 6. These STAT proteins contain between 750 and 850 amino acids and have similar structures and functions. STAT3 is composed of six different

functional regions, namely, the N-domain/STAT protein interaction domain, coiled-coil domain (CCD), DNA-binding domain (DBD), linker domain, the SH2 domain, and the Transcriptional Activation Domain (TAD) (6, 7). There are prior reviews about the involvement of STAT3 in many organs. However, the role of STAT3-related signaling pathways in liver IRI has not been systematically summarized. Therefore, we have analyzed the relevant literature with keywords such as STAT3, ischemia reperfusion injury, and liver, amongst others. This paper aimed to review the various studies of STAT3 in liver IRI and discuss its associated pathways and different roles, to provide a reference for further research.

1 Structure and function of STAT3 and the relationship between STAT3 and the mitochondria

The human STAT3 gene is located on chromosome 17q21 and encodes an 89-kDa protein (8). STAT3 mainly consists of three different isoforms, namely STAT3α, STAT3β, and STAT3γ, of which the first is the most common. STAT3α can bind to IL-6 and IL-10 secreted by macrophages. STAT3β can inhibit the synthesis of inflammatory factors and plays an anti-inflammatory role while STAT3γ is mainly produced by the degradation of STAT3α and is activated by differentiated neutrophils (9, 10).

Moreover, based on *in vitro* and *in vivo* experiments, Lucy Xi Lou showed that STAT3 knockout resulted in increased expression of transaminase and inflammatory indicators suggesting that endogenous STAT3 plays a protective role in IRI (11). However, the upstream and downstream pathways associated with STAT3 were not investigated in this study, and the specific mechanism requires confirmation by subsequent experiments. Endogenous negative regulators of STAT3, such as suppressor of cytokine-induced STAT signaling (SOCs), can bind to activated receptors and interact with Janus kinase (JAK), which in turn inhibits the activation of the STAT pathway (12). In addition, there are nuclear factors that can bind to phosphorylated STAT, commonly known as PIAS (protein inhibitors of activated STATs), of which PIAS3 is a specific inhibitor of STAT3. It can block dimerization of the STAT3 monomer or promote the dissociation of dimerized STAT3, thus inhibiting STAT3 activation (13). STAT3 has two different phosphorylation sites, namely, Tyrosine 705 (Y705) and Serine 727 (S727). STAT3 phosphorylated at Y705 dimerizes and translocates to the nucleus, while phosphorylation at S727 leads to translocation to the mitochondria (14, 15). P-STAT3 regulates the activity of the electron transport chain (ETC) through S727 (16). Mitochondria are the main sites for ROS production, and the ETC is the most important source of ATP (17). In IR, excessive ROS and Ca^{2+} can cause the opening of the mitochondrial permeability transfer pore (MPTP) and adversely affect the mitochondrial membrane potential. This, in turn, can lead to peroxidation of the mitochondrial membrane, the release of cytochrome c, the inhibition of ATP synthesis, and, finally, irreversible cell death caused by mitochondrial membrane peroxidation (18, 19). ROS can activate STAT3 during IRI (20). STAT3 can inhibit MPTP opening caused by ROS production (Figure 1), thereby reducing mitochondrial damage (21). The levels

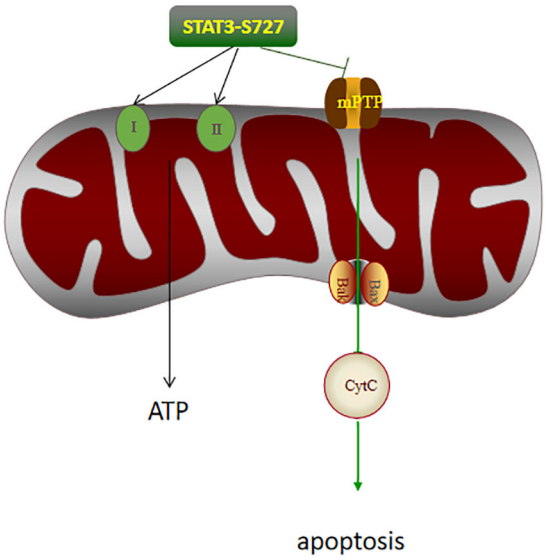

FIGURE 1
The effects of P-STAT3 on the mitochondria in hepatic IRI. P-STAT3 can promote ATP synthesis by increasing the activity of respiratory chain complex I and II. P-STAT3 also inhibited the opening of mPTP, thereby inhibiting the expression of Bax and the release of CytC, and finally alleviating apoptosis.

of P-STAT3 in the mitochondria increase rapidly during reperfusion, while the P-STAT3 level in the cytosol decreases rapidly (22). Phosphorylated STAT3 (P-STAT3) is usually present in mitochondrial inner membrane adjacent to the matrix and is important for maintaining mitochondrial integrity. GRIM-19, the main component of mitochondrial complex I, promotes the entry of P-STAT3 into the mitochondria (23).The binding of P-STAT3 to the respiratory chain increases the membrane potential and increases ATP production. STAT3 knockdown can inhibit the rate of mitochondrial respiratory chain and complex I, II activity, which can then lead to the release of excess cytochrome C, thereby aggravating IRI (24).

2 STAT3-related modes of death

Liver IRI is associated with various forms of cell death, including necrosis, apoptosis, autophagy, and ferroptosis, but those most associated with are apoptosis and autophagy. Apoptosis is a form of programmed cell death responsible for the maintenance of homeostasis in multicellular organisms (25). A number of studies have reported that 50-70% of endothelial cells and 40-60% of hepatocytes undergo apoptosis during reperfusion (26, 27). STAT3 can inhibit apoptosis in two distinct ways. First, STAT3 can play a direct anti-apoptotic role by upregulating the expression of the anti-apoptotic protein Bcl-2 and downregulating the expression of the pro-apoptotic protein Bax (28–30). Second, STAT3 can also inhibit MPTP formation to stabilize the mitochondrial membrane potential ΔΨm and thereby reduce ROS production, both of which can simultaneously inhibit the release of apoptosis-related cytokines, suppress caspase-related death pathways, attenuate the fragmentation of genetic material DNA, and ultimately inhibit apoptosis (31, 32). Autophagy is a process involved in the

degradation of proteins and organelles in cells. Autophagy-related 5 (ATG5) and Microtubule-associated protein 1 Light BII (LC3BII) are two important autophagy-related proteins in IR. Yufang Han found that STAT3 was able to activate ATG5-mediated autophagy, thereby attenuating IRI (33). Shipeng Li reported that microRNA-17 (mir-17) promoted the expression of autophagy protein LC3BII by inhibiting the expression of STAT3, and ultimately aggravated liver IRI (34). Therefore, the potential relationship between STAT3 and liver IRI reported during autophagy needs to be further explored.

3 STAT3 and liver cells

The liver is the largest parenchymal organ in the human body, and contains non-inflammatory cells such as hepatocytes and endothelial cells as well as inflammatory cells such as Kupffer cells and lymphocytes.

3.1 STAT3 and non-inflammatory cells

Hepatocytes account for 80% of the liver tissue maintaining its main metabolic functions. In the carbon tetrachloride and alcohol models of acute liver injury, the inflammatory index was found to be lower in STAT3-knockout mice, while the inflammatory index was higher in the ConA-induced hepatitis and LPS-induced models of STAT3-knockout mice. STAT3 may inhibit inflammation by inhibiting STAT1, so STAT3 can inhibit the activation of the pro-inflammatory factor STAT1 in ConA-induced and LPS-induced hepatitis (35–38). STAT3 has a dual role in hepatocytes. Model differences are one of the important reasons, which need to be further explored in other models in the future. Some researchers have found that the degree of apoptosis and increased inflammatory response in mice with endothelial-cell STAT3 knockout in the alcoholic liver model, but the specific mechanism has not been explored. There are few studies on STAT3 in endothelial cells, and further exploration of its actions is needed in the future (39).

3.2 STAT3 and inflammatory cells

Hepatic macrophages are termed Kupffer cells (KCs). KCs account for 20% to 35% of all the non-parenchymal cells in the liver and are an important component of the immune cell compartment. KCs can generate oxidative stress through regulating different pathways and stimulating the production of TNF-α and other inflammatory factors, thereby aggravating IRI (40). KCs act mainly through the recognition of Toll-like receptors (TLRs), which are important receptors involved in the inflammatory cascade (41, 42). TLR4 is the most important member of the TLR family, and STAT3 is one of its important ligands. TLR4-deficient mice have significantly reduced IRI. KCs are activated by two mechanisms (Figure 2), M1 and M2. M1 can release various inflammatory factors and cause tissue damage, whereas M2, in contrast to M1, has anti-inflammatory effects (43, 44). SS-31 is a novel antioxidant targeting mitochondria, whose main effects include promoting the

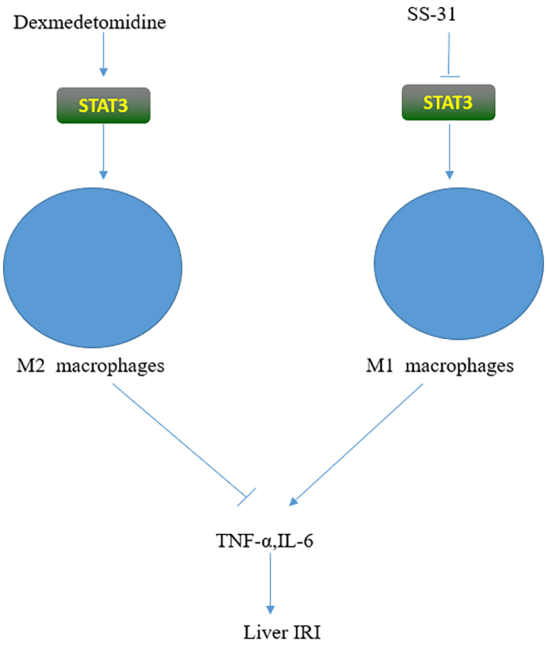

FIGURE 2
STAT3 has opposing effects on macrophages. On the one hand, it can promote the release of inflammatory factors and aggravate IRI by activating M1; on the other hand, it can activate M2, inhibit the release of inflammatory factors, thus alleviating IRI. The different effect depends on the activator of STAT3.

production of ATP and inhibiting ROS production (45, 46). Longcheng Shang reported that SS-31 could inhibit the production of mitochondrial ROS, thereby reducing the phosphorylation of STAT1 and STAT3. This can suppress the polarization of M1 macrophages, inhibit the release of inflammatory factors such as TNF-α and IL-1β, and ultimately alleviate liver IRI (47). Dexmedetomidine is a selective α2 adrenergic receptor agonist used for sedation and anesthesia in surgical patients. Haoming Zhou found that dexmedetomidine could activate the peroxisome proliferator-activated receptor-γ (PPARγ)/STAT3 pathway, thereby promoting the activation of M2 macrophages, suppressing the release of TNF-α and other inflammatory factors, and ultimately alleviating liver IRI (48–50). Zhuqing Rao reported that hyperglycemia could aggravate liver IRI by inhibiting the polarization of M2 macrophages and IL-10 activation by inhibiting STAT3 through CCAAT/enhancer-binding protein(C/EBP) protein-mediated ER stress (51). Roquin-1 is an E3 ubiquitin ligase originally identified in a mutated gene in SLE mice (52). Lei Zheng found that Roquin-1 effectively inhibited the polarization of M1 macrophages and promoted the activation of M2 macrophages, which inhibited AMP-activated protein kinase a (AMPKa) activity and promoted the activation of mammalian target of rapamycin (mTOR) and STAT3, which, in turn, led to the reduced production of related inflammatory factors and ultimately alleviated hepatic IRI (53). Tammy M found that proteolysis inducing factor (PIF) may activate STAT3 in human Kupffer cells, thereby inducing the inflammatory response. To improve the condition of patients with cachexia, inhibitors of this pathway should be further investigated (54). Lara Campana found that the STAT3-IL10-IL6 pathway promotes phenotypic transformation of human macrophages,

thereby alleviating acute liver injury (55). Ozturk Akcora STAT3 inhibitor BWP1066 inhibits the release of inflammatory cytokines from human macrophages, thereby alleviating acute liver injury (56). STAT3 is essential for the growth and development of B lymphocytes, and IL-21 secreted by T cells promotes the transformation of CD19+B cell precursors into plasma cells that secrete IgG. Leptin can promote the secretion of IL-6 and TNF-α by human B cells through activation of the JAK2/STAT3 pathway and thus aggravate the inflammatory response (57–59). STAT3 in CD8+T lymphocytes is closely related to IL-21. In CD8+T lymphocytes, IL-6 can promote the expression of STAT3, which promotes the production of IL-21 and ultimately stimulates the production of CD8+ memory cells (60). STAT3 plays an important role in Th17 cells. STAT3 promoted the secretion of the anti-inflammatory factors TGF-β1 and IL-10 by CD4+T lymphocytes, inducing more Th3 cells (61).

4 STAT3 and upstream inflammatory cytokines

KCs can secrete several inflammatory cytokines. Many inflammatory factors such as the interleukin family (ILs) can act as ligands to influence STAT3 activation. ILs that function as ligands mainly include IL-6, IL-11, and IL-22. For example, Heng Zhou reported that after vagotomy, the expression of IL-22 was decreased, and then the expression of STAT3 was reduced, inflammatory cell infiltration was increased, and IRI was aggravated. Exogenous IL-22 supplementation can promote the phosphorylation of STAT3, thereby promoting the expression of the cyclin D1 gene, and ultimately reversing liver IRI; however the mechanisms through which cyclin D1 can potentially reverse IRI in this model needs to be further explored (62). Paul J Chestovich found that IL-22 could effectively promote the phosphorylation of STAT3, inhibit the production of inflammatory factors, and ultimately reduce liver IRI but the endogenous IL-22 content was significantly increased after 24 hours of reperfusion (63). Bai, Y reported that IL-22 could activate STAT3, inhibit apoptosis and oxidative stress, and alleviate biliary IRI after liver transplantation (64). Wanzhen Li found that Ac2-26, a derivative of the endogenous inflammatory inhibitor Annexin A1 (AnxA1), can inhibit hepatocyte apoptosis induced by the mitochondrial pathway through activation of the IL-22/STAT3 pathway. Ac2-26 can also protect ATP and the mitochondrial membrane potential (MMP), inhibit MDA and ROS production, thus reducing IRI (65, 66). Nicolas Melin found that the TLR5 agonist CBLB502 can attenuate hepatic IRI by binding to the TLR5 receptor and stimulating IL-22 production by affecting the different immune cells through activation of STAT3 (67). These findings suggest that IL-22 can act as an important inflammatory factor and associate closely with STAT3, which is worthy of further study in the future. Miao Zhu showed that IL-11 could inhibit the phosphorylation of STAT3, thereby suppressing the activation of inflammatory factors such as TNF-α and IL-10, and thus attenuating liver IRI (68). Some relevant inflammatory factors play an important role in chronic liver injury, and IL-17A is a key factor in liver fibrosis. Xiao Wei Zhang found that activation of the IL-17A/STAT3 pathway can inhibit autophagy in liver cells, thus aggravating liver fibrosis, while an IL-17A inhibitor could reverse the development of fibrosis (69). Hongwei Tang added IL-6 rs1800796 into human L02 cells to activate the IL-6/STAT3 pathway, inhibit the expression of autophagy proteins, and thus reduce IRI. Recombinant human IL-6 can be a therapeutic target for hepatic IRI (70). Kun Xie found that exosomal mir-1246 derived from human umbilical cord blood mesenchymal stem cells can regulate the balance of helper/modulator T cells through the mir-1246-mediated IL-6-gp130 (IL-6 receptor)-STAT3 axis, which ultimately could attenuate liver IRI (71). Matsumoto pointed out that ischemic preconditioning (IPC) can significantly reduce hepatic IRI through activation of the IL-6-GP130-STAT3 axis, but the specific mechanisms require further exploration (72). Dayoub R found that the IL-6-STAT3-thrombopoietin (TPO) pathway can stimulate the production of megakaryocytes in the spleen and bone marrow and play a hemostatic role after acute liver injury (73). Rania Dayoub found that exogenous the acute phase response (ALR) can inhibit the IL-6/STAT3 pathway in L-02 cells, inhibit acute phase proteins (APPs) and thus ultimately inhibit inflammatory response. However, endogenous ALR activates the IL-6/STAT3 pathway and enhances the inflammatory response, so ALR has a dual role. However, the relationship between ALR and STAT3 phosphorylation needs further investigation (74). IL-6 is pleiotropic factor. As a ligand, it can play a positive role by activating the various downstream proteins. On the other hand, persistent release of IL-6 in has been implicated in various diseases. Thus, the key challenge remains to effectively balance the physiological and pathological functions of IL-6 in cells.

5 JAK - STAT3 pathway

The JAK-STAT pathway was originally discovered by Darnell et al. It is known to be an important intracellular signal transduction pathway, and has been implicated in the regulation of growth, differentiation, apoptosis, and development of various cells (Figure 3). It can promote the phosphorylation and activation of diverse proteins with tyrosine residues, generate a cascade reaction of kinase activation, and transduce the activated signal to other molecules, such as STAT, thereby triggering a series of genetic and protein changes (75, 76). In mammals, more than 40 cytokines can potentially activate the JAK -STAT pathway to influence hepatic IRI (77–79). JAK is a tyrosine kinase widely present in different types of cells, and can be divided into four types, namely, JAK1, JAK2, JAK3, and TYK2 (80). When the ligand binds to its surface receptor, it causes changes in the cytosolic part of the receptor, thus promoting the phosphorylation of the JAK protein. STAT3 and JAK are closely bound together in the resting state. When JAK is phosphorylated, STAT3 and JAK are separated to promote the phosphorylation of STAT3, and the activated STAT3 can undergo heterodimerization followed by the translocation to the nucleus, which can promote the transcriptional expression of the various genes (80–82). The STAT3 TAD contains three different sites, namely Y701, Y705, and Ser 727. Since TAD contains binding sites for its own dimer, it is quickly activated once stimulated in the cell. Exposure of Tyr705 can accelerate the process of heterodimerization, and it has been documented that increased phosphorylation of STAT3 at Tyr705 in the nucleus can reverse the inhibition of STAT3 phosphorylation at 727 during IRI (20, 83, 84).

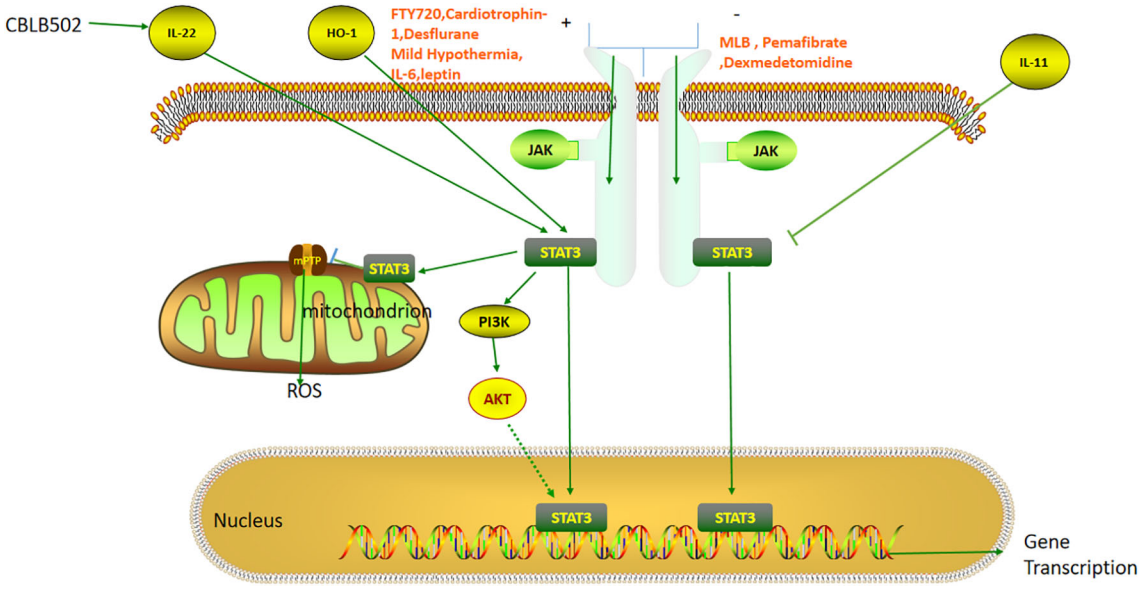

FIGURE 3
The role of the STAT3 pathway in liver ischemia-reperfusion injury. FTY720, Cardiotrophin-1, Desflurane, Mild Hypothermia, IL-6, and Leptin reduce hepatic ischemia-reperfusion injury by activating JAK-STAT3 pathway. MLB, Pemafibrate, and Dexmedetomidine can attenuate the injury by inhibiting the JAK-STAT3 pathway. IL-22, HO-1 and IL-11 can directly activate and inhibit STAT3 respectively. STAT3 can also attenuate ischemia-reperfusion injury by inhibiting mPTP opening and thereby reducing ROS release.

JAK-STAT3 activation can attenuate liver IRI. For instance, Mahmoud AR has found that coenzyme Q10 (CoQ10), which forms part of the mitochondrial respiratory chain in hepatocytes, was able to suppress apoptosis and oxidative stress by activating the JAK1/STAT3 pathway (85). Fingolimod (FTY720) is an inhibitor of the Sphingosine-1-phosphate (S1P) receptor with diverse anti-inflammatory effects (86). Xiangmin He demonstrated that Fingolimod (FTY720) could activate the JAK2/STAT3 pathway, thereby inhibiting hepatic IRI induced by acetaminophen (APAP) (87). Relevant studies have confirmed that STAT3 in hepatocytes can promote liver regeneration after hepatectomy. However, Feng D found that STAT3 had no effect after 6 h of APAP-induced ALI (88, 89) while Nishina T observed that STAT3 was still functional after 24 h; thus the specific relationship between APAP and STAT3 requires further investigation (90).The authors also reported that P-JAK2/P-STAT3 expression decreased after ischemia-reperfusion alone, which was inconsistent with previous results. The authors explained that it was related to time, and the times of IRI in this model were 1 h and 6 h, both of which were significantly shorter than those used in previous studies (91). Therefore, it is necessary to further study the activation of the pathway at the different time points in the future. Heng Chao Yu found that the Notch pathway could also activate JAK2/STAT3, promote the expression of manganese superoxide dismutase (MnSOD), inhibit ROS and apoptosis, and ultimately attenuate hepatic IRI (92). Cardiotrophin-1 was originally used as a drug to promote cardiac hypertrophy (93). Maria Iniguez reported that the myocardial nutrient cardiotrophin-1 could attenuate hepatic IPI by activating the JAK/STAT3 pathway, but the specific mechanism requires further study (94). Mengxia Zhong found that desflurane inhibited mir-135b-5p, thereby stimulating JAK2-STAT3 activation, inhibiting apoptosis, and ultimately alleviating liver IRI (95). However, there are also several reports that support the opposite conclusion. L Xiong found that mir-93 could inhibit the JAK/STAT3 pathway, thereby suppressing the production of apoptosis, inflammatory factors and transaminases, and leading to the alleviation of liver IRI (96). Ziqi Cheng reported that pemafibrate, a selective inhibitor of PPARα, could inhibit the release of inflammatory factors produced by Kupffer cells, attenuate the JAK2/STAT3β/PPARα pathway, suppress cell apoptosis as well as autophagy, and ultimately attenuate liver IRI (97). Ning Zhang found that Magnesium Lithospermate B, a traditional Chinese medicine, could markedly inhibit the production of inflammatory factors such as TNF-α and IL-6 by inhibiting the JAK2/STAT3 pathway, thereby attenuating hepatic IRI (98). Maria Cecilia S. Freitas reported that the JAK2 inhibitor AG490 could negatively regulate JAK-STAT signaling, thereby reducing the production of inflammatory factors, inhibiting apoptosis, and ultimately reducing IRI. It was also found that STAT1 activation was more likely to cause IRI than STAT3 (99). Y X Zhu found that dexmedetomidine can inhibit JAK/STAT3 signaling, apoptosis and the inflammatory response, as well as oxygen-glucose deprivation (OGD)-mediated human hepatic IRI (100). STAT1 and STAT3 have been reported to exert opposite effects on cell proliferation, differentiation, and apoptosis, which deserves further study in the future (101).

6 STAT3 and PI3K/AKT

The phosphoinositide-3 kinase (PI3K)/protein kinase B (PKB/AKT) is an important pathway involved in protein synthesis and is closely related to the regulation of redox reactions in mitochondria. PI3K/AKT can alleviate hepatic IRI by inhibiting the release of inflammatory factors and cell apoptosis while promoting autophagy (102). Bibo Ke reported that heme oxygenase-1 (HO-1) could promote the phosphorylation of STAT3, activate PI3K/AKT, inhibit the release of TNF-α and IL-10 induced by TLR-4, leading to reduced IRI (103). Bibo Ke found that STAT3 could activate PI3K/AKT by activating β-catenin, inhibiting IL-

12 and Bax, and ultimately attenuating liver IRI (104). Huang J found that the nuclear factor E2-related factor 2 (Nrf2)-HO-1 axis could activate the Notch1/Hairy and enhancer of split homolog-1(Hes1)/STAT3 pathway, promote the macrophage differentiation and PI3K/AKT pathway activation, inhibit apoptosis, and ultimately reduce liver IRI (105). Therefore, the STAT3/PI3K/AKT pathway can play a key role in acute injury but the specific downstream mechanism of PI3K/AKT needs to be further explored.

7 STAT3 and lipid metabolism

Given the increasing number of patients with nonalcoholic fatty liver disease (NAFLD), the number of patients with fatty liver disease requiring organ transplantation has increased. Fatty liver is susceptible to IRI and two different hypotheses have been proposed to account for this, namely, impaired hepatic microcirculation and mitochondrial dysfunction (Figure 4). The volume of the steatotic hepatocytes becomes larger, squeezing and narrowing the perisinusoidal space, thus increasing the resistance of hepatic microcirculation. Fatty liver can also cause mitochondrial dysfunction through promoting the production of ROS, thereby interfering with cellular energy metabolism in the liver (106, 107). STAT3 activates peroxisome proliferator-activated receptor (PPAR)γ, then up-regulates the transcription of C/EBP, and promotes the transformation of preadipocytes into adipocytes. STAT3 knockout mice showed weight gain due to hypertrophy of adipocytes, suggesting that STAT3 plays a role in lipid degradation. JAK2-STAT3 promotes lipid degradation by inhibiting the expression of fatty acid synthase and acetyl-CoA carboxylase (108, 109). In several adipose models, STAT3 knockdown in the hepatocytes was observed to aggravate steatosis (35, 110, 111). Sterol regulatory element-binding protein-1 (SREBP-1) is a transcription factor that can regulate liver lipid metabolism. STAT3 can inhibit hepatic fat accumulation by suppressing SREBP-1, and ultimately reduce hepatic steatosis (110, 112). Marco Carbone reported that the addition of leptin to the preservation solution could activate STAT3 and reduce the degree of apoptosis, thereby attenuating the development of cold IRI (113). However, the specific mechanism of STAT3 in liver cold preservation needs to be further explored. Renalase is a ubiquitous pan-xanthine dinucleotide amine oxidase found in many organs (114). Tao Zhang reported that renalase could activate the STAT3-SIRT1 pathway and inhibit IRI in fatty liver (17). Zhihui Jiao found that the secretory proteome of adipose-derived mesenchymal stem cells could inhibit the expression of SOC3 and the negative feedback effect of SOC3 on STAT3 can lead to increase the expression of P-STAT3, and reduce IL-6, TNF-α and other related inflammatory factors, thereby alleviating liver IRI (115). Euno Choi found that P-STAT3 might aggravate liver steatosis and inflammatory injury, which was the first time for P-STAT3 to be explored in specimens of patients with fatty liver disease. The authors did not clarify whether this effect was related to the leptin pathway, and further research is needed to explore the relationship (116).Therefore, STAT3 plays different roles in regulating the process of lipid metabolism, and future studies to investigate its role in fatty liver are warranted.

8 Conclusion

In hepatic IRI, STAT3 usually binds to the mitochondria to regulate programmed cell death. STAT3 plays a role in many hepatic cells. The STAT3-associated IR pathway includes upstream cytokines, and JAK, and downstream PI3K/AKT. The role of STAT3 in liver IRI is controversial (Table 1). On the one

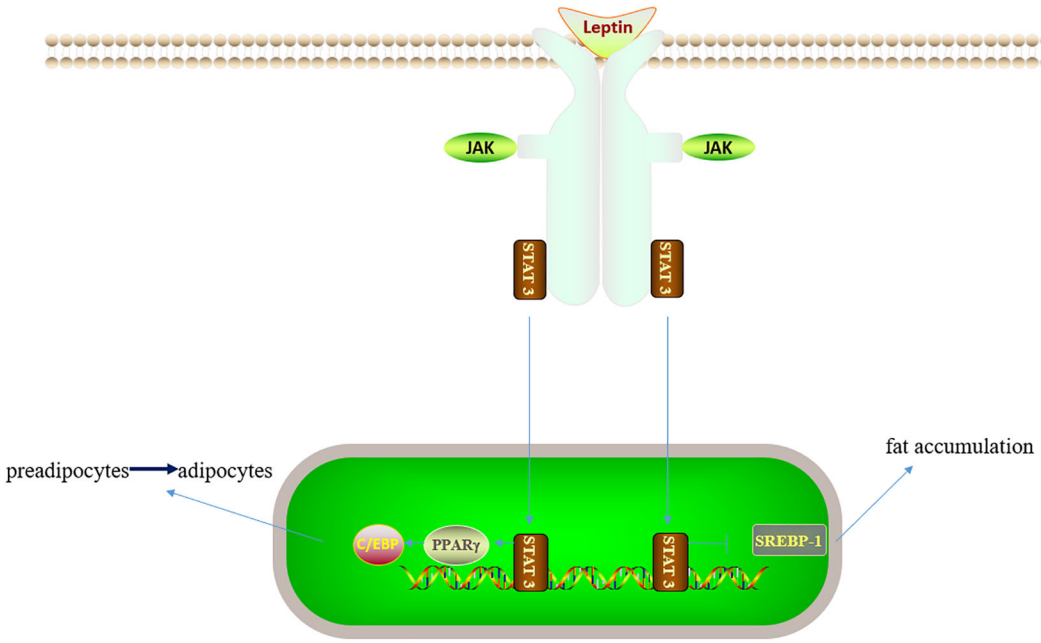

FIGURE 4
Leptin/JAK/STAT3 related lipid metabolic pathway. On the one hand, STAT3 can activate PPARγ, C/EBP and inhibit liver adipocyte maturation; on the other hand, STAT3 can inhibit SREBP-1 and thus inhibit fat accumulation. Therefore, STAT3 has different roles in lipid metabolism.

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TABLE 1 An overview of the role of STAT3 in hepatic ischemia-reperfusion.

Author	Journal	Year	Species	Finding
Iñiguez M	J Exp Med	2006	mouse	Cardiotrophin-1 alleviates hepatic IRI by activating the JAK/STAT3 pathway.
Yu HC	Hepatology	2011	mouse	The Notch pathway can activate the JAK2/STAT3 pathway, promote the expression of manganese superoxide dismutase (MnSOD), ultimately alleviate hepatic IRI.
Ke B	J Hepatol	2012	mouse	HO-1 promotes STAT3 phosphorylation, activating PI3K/AKT thereby alleviating liver IRI.
Carbone TM	Transpl Int	2012	rat	Leptin can activate STAT3 and reduce the degree of necrosis and apoptosis, thereby alleviating cold IRI.
Chestovich PJ	Transplantation	2012	mouse	IL-22 can promote the phosphorylation of STAT3, inhibit the production of inflammatory factors, and ultimately alleviate liver IRI.
Ke B	Hepatology	2013	mouse	STAT3 can activate β-catenin followed by activation of PI3K/AKT, and ultimately alleviate hepatic IRI.
Huang J	Mol Med	2014	mouse	The Notch1/Hes1/Stat3 pathway promotes the activation of PI3K/AKT pathway, ultimately alleviating liver IRI.
Zhu M	PLoS One	2015	mouse	IL-11 can inhibit the phosphorylation of STAT3, thereby inhibiting the activation of inflammatory factors, and ultimately alleviating liver IRI.
Li S	Liver Transpl	2016	mouse	mir-17 promotes the expression of LC3BII by inhibiting the expression of STAT3, and ultimately aggravates hepatic IRI.
Rao Z	Front Immunol	2017	mouse	Hyperglycemia can inhibit STAT3 through C/EBP protein mediated ER stress, thus aggravating liver IRI.
Han YF	J Cell Biochem	2018	mouse	STAT3 can activate ATG5 protein-mediated autophagy, thereby alleviating IRI.
Zhu YX	Eur Rev Med Pharmacol Sci	2018	human	Dexmedetomidine can inhibit the JAK/STAT3 pathway, and ultimately inhibit oxygen-glucose deprivation (OGD) -mediated IRI.
Mahmoud AR	Tissue Cell	2019	rat	CoQ10 inhibits apoptosis and oxidative stress by activating the JAK1/STAT3 pathway.
Xie K	IUBMB Life	2019	mouse	Mir-1246 regulates IL-6-GP130 -STAT3 axis, and ultimately alleviates hepatic IRI.
Zhang N	Front Pharmacol	2019	mouse	MLB could inhibit the JAK2/STAT3 pathway, thus inhibiting liver IRI.
Zhang T	Oxid Med Cell Longev	2019	mouse	Renalase can activate the STAT3-SIRT1 pathway and inhibit IRI of fatty liver.
Zhu YX	Int Immunopharmacol	2020	mouse	Dexmedetomidine can activate the PPARγ/STAT3 pathway, ultimately alleviating liver IRI.
Zheng L	J Immunol	2020	mouse	Roquin 1 inhibits the activity of AMPKa and promotes the activation of mTOR and STAT3, thereby alleviating liver IRI.
Wang W	Oxid Med Cell Longev	2020	mouse	Mild hypoxia was found to activate the JAK2-STAT3-CPT1A pathway, ultimately promoting the β -oxidation of fatty acids, and ultimately alleviating liver IRI.
Ozturk A	FASEB Bioadv	2020	human	BWP1066 inhibits the release of inflammatory cytokines from macrophages thereby alleviating hepatic IRI.
Shang L	Oxid Med Cell Longev	2021	mouse	SS-31 can inhibit the STAT3, ultimately alleviating liver IRI.
Zhong M	J Chin Med Assoc	2021	rat	Desflurane can inhibit mir-135b-5p to promote the activation of JAK2-STAT3, ultimately alleviating liver IRI.
Melin N	Cell Death Dis	2021	mouse	CBLB502 alleviates hepatic IRI through the IL-22-STAT3 pathway.
Jiao Z	Stem Cell Res Ther	2021	pig	The adipose-derived mesenchymal stem cell secretome inhibit the expression of SOC3, increase the expression of P-STAT3, to alleviate liver IRI.
Zhou H	J Immunol Res	2021	mouse	IL-22 can promote the phosphorylation of STAT3, which in turn promotes the expression of cyclinD1, and ultimately alleviates liver IRI.
Cheng Z	PPAR Res	2021	mouse	Pemafibrate can inhibit JAK2/STAT3β/PPARα pathway, ultimately alleviating liver IRI.
Tang H	Mol Biol Rep	2021	human	Recombinant human IL-6 can activate STAT3, inhibit autophagy proteins, and ultimately alleviate liver IRI.
He X	Int J Mol Med	2022	mouse	FTY720 activates the JAK2/STAT3 pathway to inhibit hepatic IRI induced by APAP.
Li W	Peer J	2022	mouse	Ac2-26 can protect ATP, mitochondrial membrane potential (MMP), and ultimately reduce IRI.

hand, STAT3 can play a protective role through the modulation of various proteins, inflammatory factors, and cells while on the other hand, it can aggravate IRI. The reason can be partly attributed to the fact that that P-Janus kinase (P-JAK) can activate both STAT3 and STAT1, and STAT3 can inhibit apoptosis whereas STAT1 can promote apoptosis. The JAK-specific inhibitor AG490 can inhibit both, thus producing different effects, but these are closely related to the length of the model time (117). The same protein may have different effects at different times and the same inflammatory factors can play diverse roles. STAT3 can also play a dual role in the regulation of lipid metabolism. On the one hand, STAT3 can promote the maturation of adipocytes while, on the other hand, it can promote lipolysis. STAT3 may also affect the microcirculation and energy metabolism by influencing fat accumulation. Therefore, STAT3 has an important effect on IRI in fatty liver. The incidence of fatty liver is increasing and it is necessary to further explore the functions of STAT3 in adipocyte maturation and lipolysis. Thus, further analysis of STAT3-related pathways in liver IRI is needed to provide a foundation for clinical treatment.

Author contributions

HY, YZ, PZ contributed to manuscript writing and editing. HY, YZ, PZ, QW, KC conducted a critical revision of the manuscript. All authors contributed to the article and approved the submitted version.

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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.

Publisher's note

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Efficacy and safety of Iguratimod in the treatment of Ankylosing Spondylitis: A systematic review and meta-analysis of randomized controlled trials

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Objective: To explore the efficacy and safety of Iguratimod (IGU) intervention in the treatment of Ankylosing Spondylitis (AS).

Methods: We used computer to search literature databases, collected randomized controlled trials (RCTs) related to IGU treatment of AS, and searched the relevant literature in each database until Sep. 2022. Two researchers independently carried out literature screening, data extraction, and evaluation and analysis of the risk of bias in the included studies, and then used Rev Man5.3 software for meta-analysis. The protocol is CRD42020220798.

Results: A total of 10 RCTs involves in 622 patients were collected. The statistical analysis showed that IGU can decrease the BASDAI score (SMD -1.62 [-2.20, -1.05], P<0.00001. Quality of evidence: low), the BASFI score (WMD -1.30 [-1.48, -1.12], P<0.00001. Quality of evidence: low) and the VAS (WMD -2.01 [-2.83, -1.19], P<0.00001. Quality of evidence: very low). Meanwhile, the addition of IGU into the conventional therapy would not increase the adverse events (RR 0.65 [0.43, 0.98], P=0.04. Quality of evidence: moderate).

Conclusion: IGU may be an effective and safe intervention for AS.

Systematic review registration: https://www.crd.york.ac.uk/prospero/display_record.php?, identifier CRD42020220798.

KEYWORDS

Iguratimod, ankylosing spondylitis, systematic review, meta-analysis, randomized controlled trial

1 Introduction

Ankylosing Spondylitis (AS) is a chronic inflammatory autoimmune disease, which mainly involves axial joint involvement, which may be accompanied by extra-articular manifestations. In severe cases, spinal deformity and joint stiffness may occur. One of the current features of AS is the high prevalence rate (0.86% in Western European white population) and the low incidence rate (1, 2). Patients can live with the disease for many years, and fusion of the spine or peripheral joints can occur in the late stage, causing the patient to lose motor function and living ability, and bring a heavy economic burden to the family and society (3, 4). Inflammation and pathological new bone formation are the two most important pathological features of AS. The early stage of AS is mainly manifested by inflammation and the bone erosion and destruction caused by it, and the late stage causes ectopic new bone formation (4, 5). As the initiating factor, inflammation runs through the entire process of disease development. There are many studies on it at present. The research on pathological new bone formation and the development of corresponding therapeutic drugs are still in the initial stage (5).

The therapeutic drugs for AS currently used clinically mainly include non-steroidal anti-inflammatory drugs (NSAIDs) and biological agents (TNF-α blockers). Although these drugs have achieved good anti-inflammatory effects, they have certain limitations and side effects, and there is no clear evidence for the role of AS new bone formation (6, 7). NSAIDs, as the first-line drugs recommended by AS treatment guidelines, have good anti-inflammatory and analgesic effects, but they need to be taken for a long time and have side effects such as cardiovascular, gastrointestinal and renal toxicity (8, 9). Similarly, TNF-α blockers are not effective for some patients, they are also very expensive, and there are reports that they may increase the risk of cancer (10). Therefore, new drugs for the treatment of AS are urgently needed clinically.

Iguratimod (IGU) is a new type of small molecule anti-rheumatic drug, which has the effects of non-steroidal anti-inflammatory drugs (NSAID) and disease mitigating anti-rheumatic drugs (DMARD). At present, it has been widely used clinically in China and Japan for the treatment of rheumatoid arthritis (RA) (11). IGU not only inhibits related inflammation-related signaling pathways and the expression of inflammatory factors (NF-κB and IL-17 inflammatory signaling pathways) (12), but also inhibits osteoclast differentiation (RANKL signaling pathway), promote osteoblast function (BMP/Dlx5/Osterix signaling pathway), and reduce cartilage destruction (MMPs family related factors) (13, 14), so as to play a bone protection role. At present, clinical randomized controlled trial (RCT)s showed the efficacy of IGU on AS (15–24), but there is no relevant research to systematic review and meta-analyze these RCTs to provide new evidence. Therefore, this research will evaluate the effectiveness and safety of IGU intervention in AS through systematic reviews and meta-analysis for the first time, in order to provide new evidence for clinical use.

2 Materials and methods

2.1 Protocol

This systematic review and meta-analysis were conducted strictly in accordance with the protocol [CRD42020220798 (see Supplementary Material)].

2.2 Search criteria

(1) Participants: Patients diagnosed with AS. All patients are at least 18 years old, and there are no restrictions on gender, race, and region. (2) Intervention methods: The intervention of the experimental group is IGU, used alone or in combination with the control group's drugs. The intervention of the control group was conventional therapy. (3) Outcomes: Bath Ankylosing Spondylitis Disease Activity Index (BASDAI), Bath Ankylosing Spondylitis Functional Index (BASFI), VAS and adverse events; secondary outcomes are ESR, CRP, TNF-α, back pain score, SOD, CTX-I, β-CTX, OPG. (4) Study design: Randomized controlled trial without any limitations.

2.3 Literature search and screening strategy

We searched the ClinicalTrials, the China National Knowledge Infrastructure Databases (CNKI), Web of Science, Pubmed, The Chinese Science and Technology Periodical Database (VIP), EMBASE, Wan Fang Database, CiNii Research, J-STAGE, National Diet Library Digital Collections (NDLDC), Chinese Biomedical Database (CBM), Medline Complete, Cochrane Library. The retrieval time is up to Sep. 2022. The search strategy was shown in Table S1. All included studies were screened by two researchers according to the search criteria. If there is a disagreement between the two, the two researchers will discuss and resolve with the other researchers.

2.4 Data extraction and quality assessment

In order to collect the sample size, baseline conditions, treatment plan, treatment time, outcomes and other information included in the RCTs, a table was made to facilitate the extraction of relevant data and retrieval records. Data extraction was carried out independently by two researchers, and differences were resolved through discussions with other researchers. RCT quality assessment is carried out according to the risk of bias tool included in the Cochrane Handbook or Systematic Reviews of Interventions Version (25). The following aspects are evaluated for each study: random sequence generation and allocation hiding (selection bias), blinding (performance bias and detection bias), incomplete outcome data (detection bias), selective reporting (reporting bias) and other bias. The results of the analysis are divided into: "yes" (low risk of bias), "no" (high risk of bias), and "unclear" (unknown risk of bias).

2.5 Statistical analysis

Review Manager 5.3 software was used for statistical analysis (26). For continuous variables such as BASDAI, BASFI, VAS, ESR, CRP, the mean difference (MD) was used to describe the effect size, and the confidence interval (CI) is 95%. For dichotomous variables such as adverse event indicators, relative risk (RR) was used to describe the impact, and the CI is set to 95%. The χ^2 test was used to analyze the heterogeneity between the results. In the case of low heterogeneity (P>0.1, I2<50%), a fixed effects model analysis was performed. If there is heterogeneity between the studies, a random effects model was used. The publication bias was detected by STATA 15 with Egger method (continuous variable) and Harbord methods (dichotomous variable) for primary outcomes. P>0.1 is considered to have no publication bias.

3 Results

3.1 Results of the search and description of included trials

The total records identified through database searching and other sources were 53. Forty (40) were excluded based on the title and abstract and 13 for more detailed evaluation. Three (3) of 13 records were excluded because they were not RCTs (27–29) (Figure 1). All patients in those RCTs come from China and involves in 622 participants. The age range of patients is 20-50 years old, and the course of treatment is at least 12 weeks and the maximum is 24 weeks. The details of study characteristics are presented in Table 1.

3.2 Risk of bias of included studies

The summary and graph of risk of bias ware shown in Figures 2, 3.

3.2.1 Sequence generation and allocation concealment

The random sequences of all RCTs are generated by random number table method, so we evaluate them as low risk of bias. Meanwhile, only Yang et al. (21) describe an acceptable method of allocation concealment, while other RCTs did not describe an acceptable method of allocation concealment. Therefore, Yang et al. (21) were rated as having a low risk of bias, while others were rated as having an unclear risk of bias.

3.2.2 Blinding

Zeng et al. (20) and Yang et al. (21) stated in the RCT that the blind method was used, but did not describe the specific

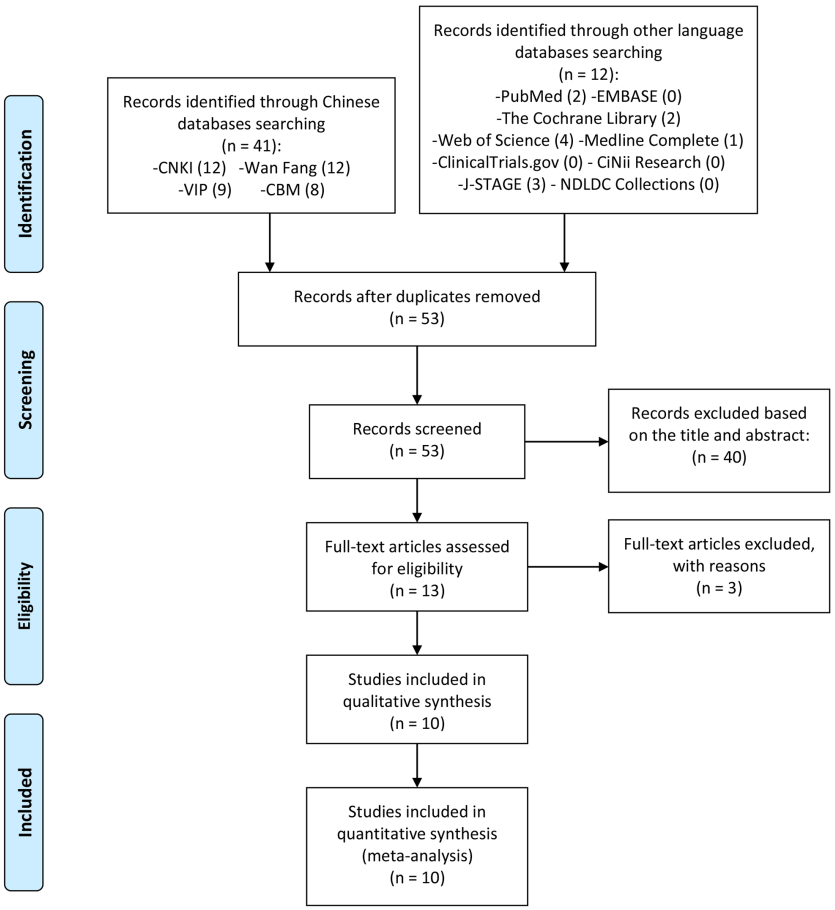

FIGURE 1
Flow diagram.

TABLE 1 The characteristics of the included studies.

Study	Country	Sample size		Intervention		Relevant outcomes	Mean age (years)		BASDAI		BASFI		Duration
		Trial group	Control group	Trial group	Control group		Trial group	Control group	Trial group	Control group	Trial group	Control group	
Qiu et al., 2016 (15)	China	18	18	Iguratimod 25mg B.i.d	NSAIDs+DMARDs	ESR, BASDAI, BASFI, VAS, back pain score, adverse events	37.3 ± 7.0	34.5 ± 9.3	5.4 ± 1.1	5.6 ± 1.1	4.9 ± 1.9	5.0 ± 1.7	24 weeks
Yuan et al., 2020 (16)	China	41	39	Iguratimod 25mg B.i.d + Etoricoxib tablets 60 mg Q.d. + ibuprofen 300 mg T.i.d. + methotrexate 15 mg once a week	Etoricoxib tablets 60 mg Q.d. + ibuprofen 300 mg T.i.d. + methotrexate 15 mg once a week	VAS, CRP, ESR, SOD, CTX-I, adverse events	39.28 ± 5.30	40.08 ± 5.67	–	–	–	–	12 weeks
Pang et al., 2020 (17)	China	39	39	Iguratimod 25mg B.i.d + Etanercept 25mg tiwce a week	Etanercept 25mg tiwce a week	ESR, CRP, BASDAI, β-CTX, OPG, TNF-α	24.85 ± 4.18	25.01 ± 4.29	6.22 ± 1.38	6.19 ± 1.28	–	–	12 weeks
Lin et al., 2019 (18)	China	24	24	Iguratimod 25mg B.i.d + Sulfasalazine 1 g B.i.d. + methotrexate 10 mg once a week + NSAIDs	Sulfasalazine 1 g B.i.d. + methotrexate 10 mg once a week + NSAIDs	BASDAI, BASFI, VAS, adverse events	32. 71 ± 8. 80	28. 21 ± 6. 69	5. 25 ± 1. 03	5. 29 ± 1. 02	4. 85 ± 1. 56	4. 62 ± 1. 34	24 weeks
Xu et al., 2019 (19)	China	21	21	Iguratimod 25mg B.i.d + Celecoxib 0.2 g Q.d.	Sulfasalazine 1 g B.i.d. + Celecoxib 0.2 g Q.d.	BASDAI, BASFI, VAS, ESR, CRP, adverse events	35.1± 10.3	34.3± 9.5	5.5± 0.9	5.6± 0.9	5.9± 1.5	6.1± 1.3	24 weeks
Zeng et al., 2016 (20)	China	25	25	Iguratimod 25mg B.i.d + Meloxicam 7.5 mg Q.d.	Sulfasalazine 0.75 g T.i.d. + Meloxicam 7.5 mg Q.d.	BASDAI, TNF-α, CRP, adverse events	38 ± 12	40 ± 10	6.21 ± 1.45	6.34 ± 1.19	–	–	24 weeks
Yan et al., 2021 (21)	China	48	25	Iguratimod 50mg Q.d + NSAIDs	NSAIDs + Placebo	BASDAI, BASFI, CRP, ESR, adverse events	31.38 ± 7.36	30.28 ± 5.94	4.69 ± 0.94	4.57 ± 0.57	3.41 ± 1.33	3.49 ± 1.23	24 weeks
Bai et al., 2021 (22)	China	43	43	Iguratimod 25mg B.i.d + Sulfasalazine 1g B.i.d + Celecoxib 200mg B.i.d	Sulfasalazine 1g B.i.d + Celecoxib 200mg B.i.d	BASDAI, VAS, CRP, ESR, adverse events	28.52 ± 9.43	27.87 ± 8.05	5.92 ± 0.96	5.88 ± 1.06	–	–	12 weeks
Li et al., 2021 (23)	China	30	30	Iguratimod 25mg B.i.d + Sulfasalazine 0.5 to 1g B.i.d + Thalidomide 50 to 200mg Qn	Sulfasalazine 0.5 to 1g B.i.d + Thalidomide 50 to 200mg Qn	BASDAI, TNF-α	31.24 ± 4.71	30.01 ± 4.68	37.47 ± 4.06	38.14 ± 4.37	–	–	24 weeks
Zhang et al., 2022 (24)	China	35	34	Iguratimod 25mg B.i.d + Celecoxib 0.2g Q.d. + Sulfasalazine 0.25 B.i.d	Celecoxib 0.2g Q.d. + Sulfasalazine 0.25 B.i.d	BASFI, CRP, ESR, SOD, CTX-I, TNF-α, adverse events	49~75	48~74	–	–	5.93 ± 1.41	5.89 ± 1.37	12 weeks

"-" indicates no data. "+" means plus.

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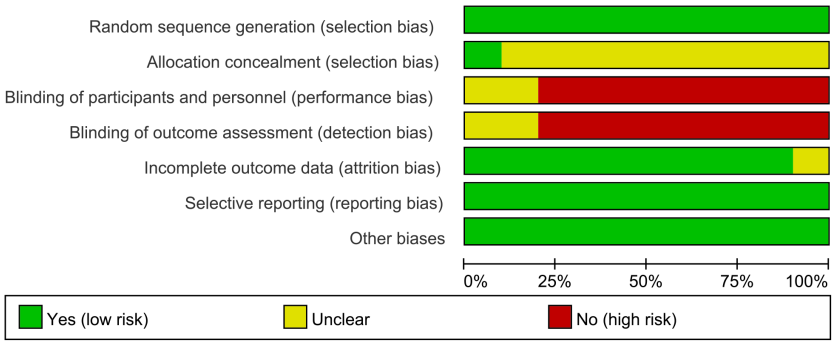


FIGURE 2
Risk of bias graph.

implementation process of the blind method, so we thought its risk of bias is unclear. Other studies did not specify whether to use blinding, and their main outcome are subjective evaluation indicators (such as BASDAI, BASFI, VAS), which are easily affected by non-blinding, so we believe that their risk of bias is high.

3.2.3 Incomplete outcome data and selective reporting

All RCTs do not have incomplete outcome data and selective reporting, so we evaluate them as low risk.

3.2.4 Other potential bias

Other sources of bias were not observed in 8 RCTs; therefore, the risks of other bias of the RCTs were low.

33 Primary outcomes

3.3.1 BASDAI

Eight RCTs (15, 17–23) utilized BASDAI to assess the improvement of AS, which include 247 patients in IGU group and 225 patients in control group. The heterogeneity test showed that P<0.00001, I2 = 86%, which suggest that the heterogeneity is high, and the random effects model was used for analysis. The meta-analysis results show that compared with the control group, the BASDAI in the IGU group was lower (SMD -1.62 [-2.20, -1.05], P<0.00001; random effect model) (Figure 4).

3.32 BASFI

Five RCTs (15, 18, 19, 21, 24) utilized BASFI to assess the improvement of AS, including 146 patients in IGU group and 122 patients in control group. The heterogeneity test showed that P=0.27, I2 = 23%, which suggest that the heterogeneity is low, and the fixed effects model was used for analysis. The meta-analysis results show that compared with the control group, the BASFI in the IGU group was lower (WMD -1.30 [-1.48, -1.12], P<0.00001; fixed effect model) (Figure 5).

333 VAS

Four RCTs (15, 16, 18, 19, 22) utilized VAS to assess the improvement of AS, including 137 patients in IGU group and 135 patients in control group. The heterogeneity test showed that P<0.00001, I2 = 95%, which suggest that the heterogeneity is high, and the random effects model was used for analysis. The meta-analysis results show that compared with the control group, the VAS in the IGU group was lower (WMD -2.01 [-2.83, -1.19], P<0.00001; random effect model) (Figure 6).

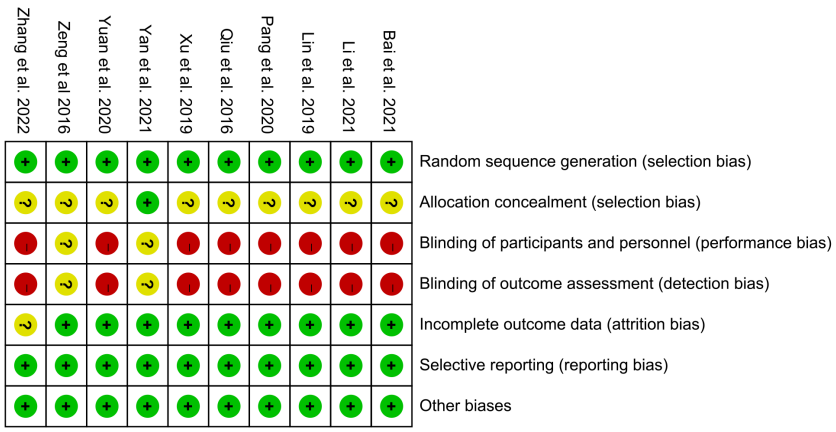


FIGURE 3
Risk of bias summary.

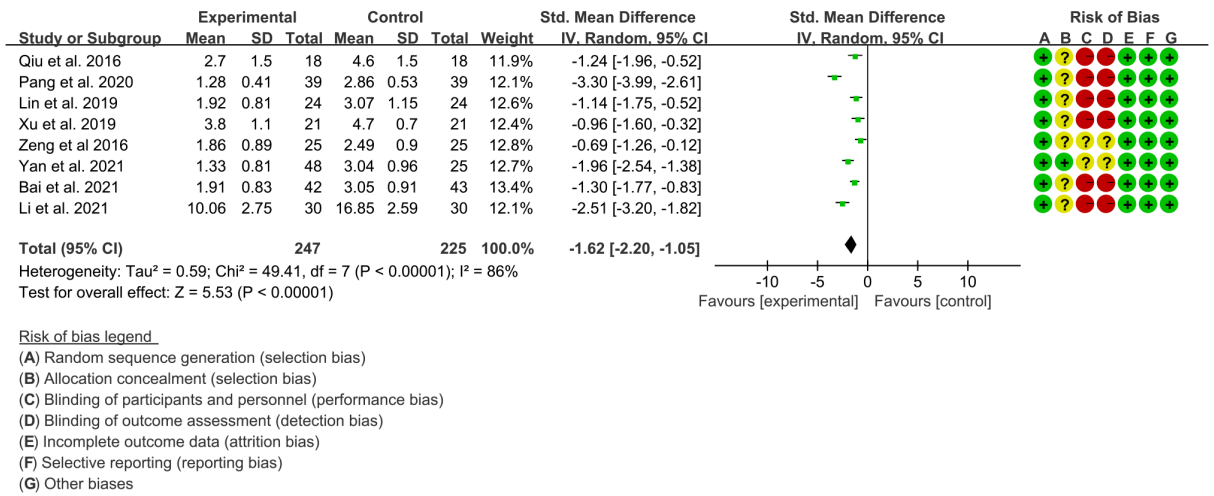


FIGURE 4
The results of BASDAI (CI, confidence interval; SD, standard deviation).

3.4 Secondary outcomes

3.4.1 The results of ESR

Six RCTs (15–17, 19, 21, 22) utilized ESR to assess the improvement of AS, which involves in 209 patients in IGU group and 185 patients in control group. The heterogeneity test showed that P<0.00001, I2 = 90%, which suggest that the heterogeneity is high, and the random effects model was used for analysis. The meta-analysis results show that compared with the control group, the ESR in the IGU group was lower (WMD -10.01 [-14.72, -5.29], P<0.0001; random effect model) (Figure 7).

3.4.2 The results of CRP

Seven RCTs (16, 17, 19–22, 24) utilized CRP to assess the improvement of AS, which involves in 251 patients in IGU group and 226 patients in control group. The heterogeneity test showed that P<0.00001, I2 = 99%, which suggest that the heterogeneity is high, and the random effects model was used for analysis. The meta-analysis results show that compared with the control group, the CRP in the IGU group was lower (WMD -10.11 [-14.55, -5.66], P<0.00001; random effect model) (Figure 8).

3.4.3 The results of TNF-α

Four RCTs (18, 21, 23, 24) utilized TNF-α to assess the improvement of AS, which involves in 129 patients in IGU group and 128 patients in control group. The heterogeneity test showed that P<0.00001, I2 = 94%, which suggest that the heterogeneity is high, and the random effects model was used for analysis. The meta-analysis results show that compared with the control group, the TNF-α in the IGU group was lower (WMD -6.21 [-7.96, -4.47], P<0.00001; random effect model) (Figure 9).

3.4.4 The results of SOD

Two RCTs (16, 24) utilized SOD to assess the improvement of AS, which involves in 76 patients in IGU group and 73 patients in control group. The heterogeneity test showed that P<0.00001, I2 = 95%,

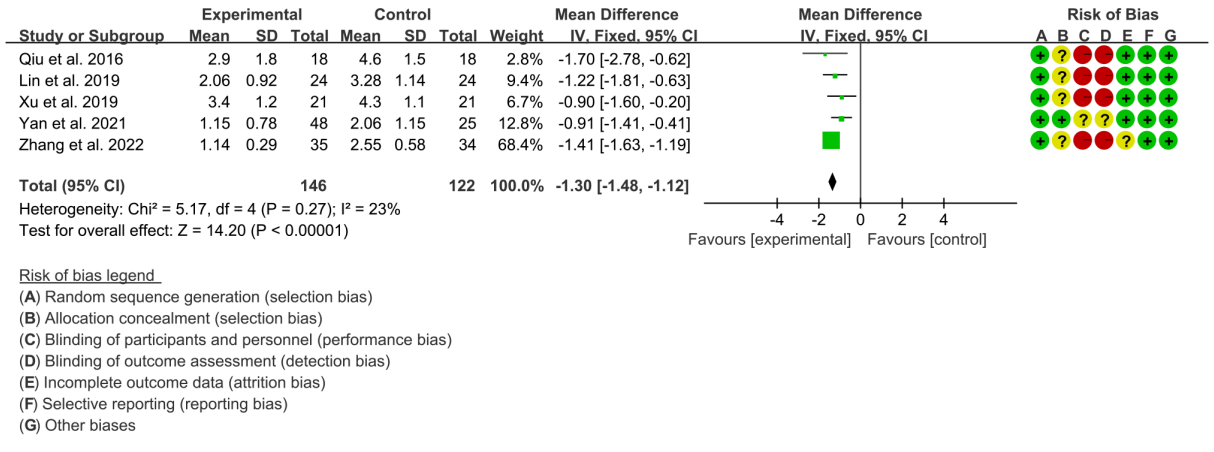


FIGURE 5
The results of BASFI (CI, confidence interval; SD, standard deviation).

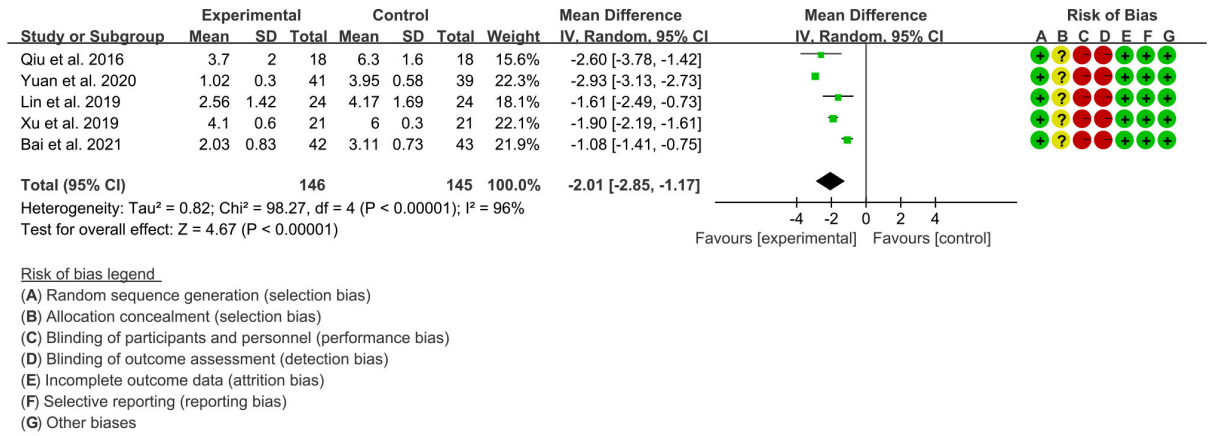


FIGURE 6
The results of VAS (CI, confidence interval; SD, standard deviation).

which suggest that the heterogeneity is high, and the random effects model was used for analysis. The meta-analysis results show that there was no significant difference in SOD between the experimental group and the control group (WMD 3.97 [-42.07, 50.01], P=0.87; random effect model) (Figure 10).

3.4.5 The results of CTX-I

Two RCTs (16, 24) utilized CTX-I to assess the improvement of AS, which involves in 76 patients in IGU group and 73 patients in control group. The heterogeneity test showed that P<0.0001, I2 = 94%, which suggest that the heterogeneity is high, and the random effects model was used for analysis. The meta-analysis results show that there was no significant difference in CTX-I between the experimental group and the control group (WMD -0.29 [-0.60, 0.01], P=0.06; random effect model) (Figure 11).

3.4.6 Other outcomes

Only Qiu et al. (16) reported back pain score, and they found that IGU can improve back pain score (P<0.05). Only Pang et al. (18) reported β-CTX and OPG levels, and they found that IGU can reduce β-CTX level and increase OPG level (P<0.05).

3.5 Adverse events

Nine RCTs (15–22, 24) (284 patients in experimental group and 258 patients in control group) reported adverse events. The heterogeneity test P=0.37, I2 = 8%, indicating that the included studies are heterogeneous, and the fix effects model is used for analysis. The results of meta-analysis showed that incidence of adverse events in IGU group was lower (RR 0.65 [0.43, 0.98], P=0.04; fix effect model) (Figure 12).

3.6 Publication bias detection

The publication bias of the primary outcomes was detected by STATA 15.0. (1) BASDAI: The publication bias detection suggests that the possibility of publication bias was small (P=0.302)

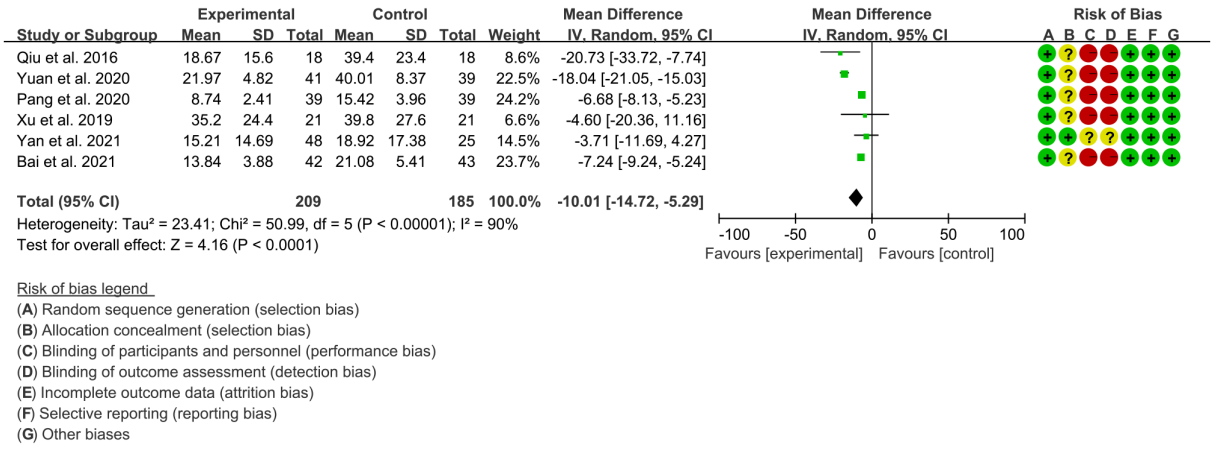


FIGURE 7
The results of ESR (CI, confidence interval; SD, standard deviation).

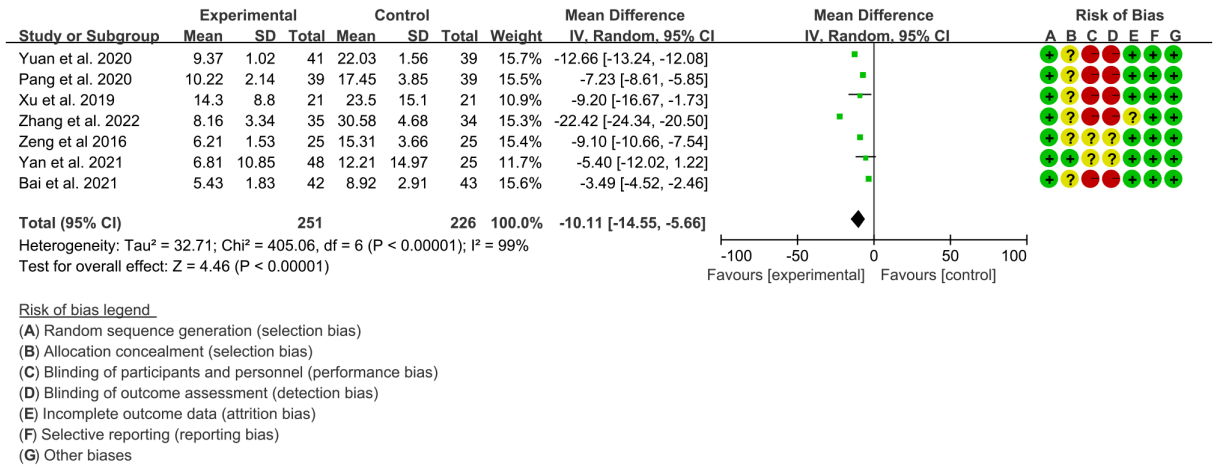


FIGURE 8
The results of CRP (CI, confidence interval; SD, standard deviation).

(Figure 13A). (2) BASFI: The publication bias detection suggests that the possibility of publication bias was small (P=0.420) (Figure 13B). (3) VAS: The publication bias detection suggests that the possibility of publication bias was small (P=0.531) (Figure 13C). (4) Adverse events: The publication bias detection suggests that the possibility of publication bias was small (P=0.844) (Figure 13D).

3.7 Subgroup analysis

The subgroup analysis was performed according to the duration (Table 2). The results of subgroup analysis showed that BASDAI, VAS, CRP, and TNF-α improved after 12 weeks of IGU treatment, and also improved after 24 weeks of treatment. However, for ESR, the addition of IGU treatment improved ESR at 12 weeks, while 24 weeks after the intervention showed no significant difference in ESR compared with the control group. For adverse events, the results showed that the 12-week intervention did not lead to an increase in the occurrence of adverse events, and the adverse events of long-term use (24 weeks) may be lower than that of the control group.

4 Discussion

This research included 10 RCTs with 622 participants. In addition to ClinicalTrials.gov, we also searched the Chinese Clinical Trial Registry and found that currently ongoing randomized controlled studies are: ChiCTR1800019227 and ChiCTR2000029112. The meta-analysis results showed that IGU can decrease the BASDAI score, BASFI score and VAS. IGU can also reduce inflammation levels (decreasing ESR, CRP and TNF-α). Most of the results are highly heterogeneous, especially VAS, ESR, CRP and TNF-α. It may be because both BASDAI and VAS are subjective measurement indicators, and the subjective feelings of patients with different RCTs are not uniform. ESR, CRP and TNF-α are individual biochemical indicators, and patients in different RCTs are different due to different conditions. All studies reported adverse

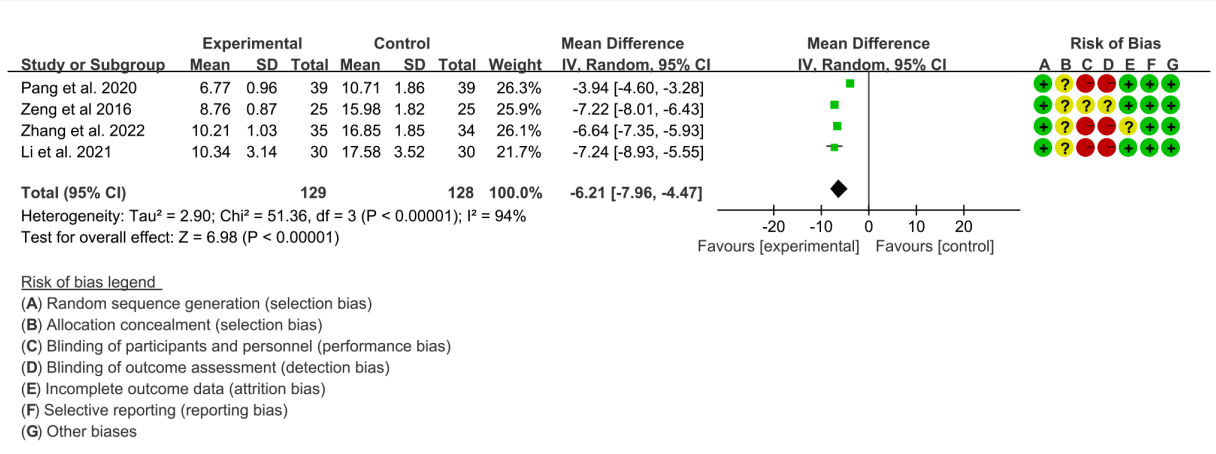
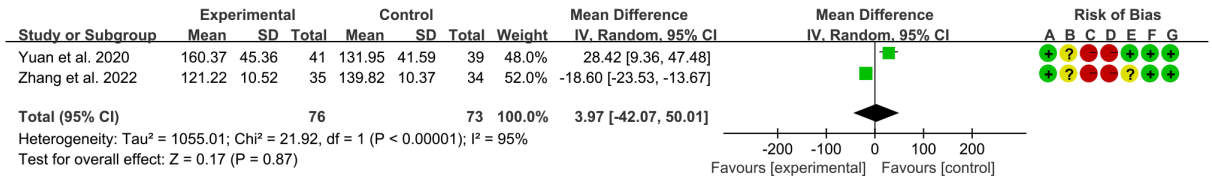
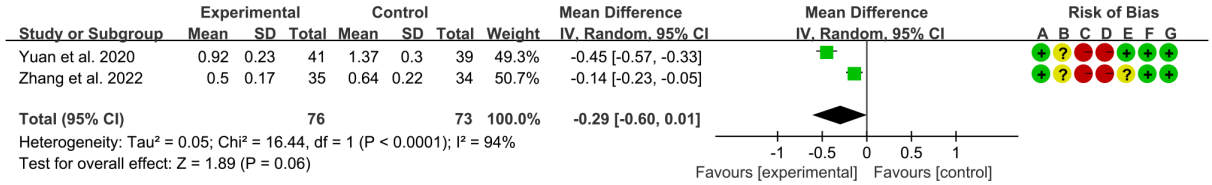


FIGURE 9
The results of TNF-α (CI, confidence interval; SD, standard deviation).



Risk of bias legend
(**A**) Random sequence generation (selection bias)
(**B**) Allocation concealment (selection bias)
(**C**) Blinding of participants and personnel (performance bias)
(**D**) Blinding of outcome assessment (detection bias)
(**E**) Incomplete outcome data (attrition bias)
(**F**) Selective reporting (reporting bias)
(**G**) Other biases

FIGURE 10
The results of SOD (CI, confidence interval; SD, standard deviation).



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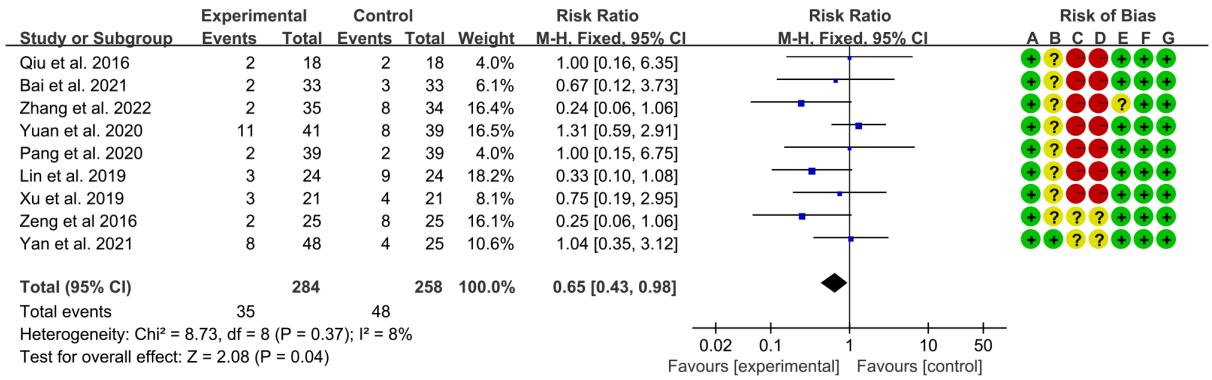
(**F**) Selective reporting (reporting bias)
(**G**) Other biases

FIGURE 11
The results of CTX-I (CI, confidence interval; SD, standard deviation).



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(**E**) Incomplete outcome data (attrition bias)
(**F**) Selective reporting (reporting bias)
(**G**) Other biases

FIGURE 12
Adverse events (CI, confidence interval; SD, standard deviation).

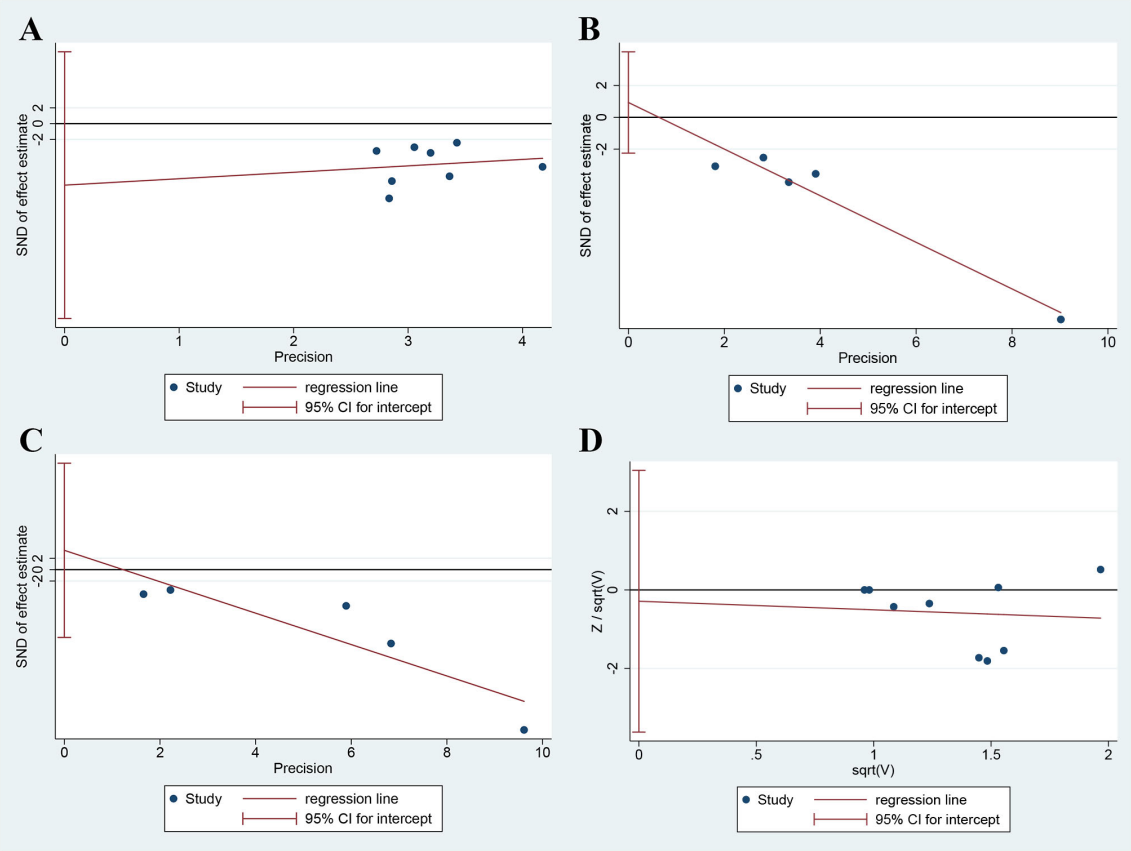

FIGURE 13
Publication Bias Detection **(A)** BASDAI; **(B)** BASFI; **(C)** VAS; **(D)** Adverse events.

reactions and no patient deaths were reported. Compared with the control group, the adverse events of the IGU group was lower. This shows that the addition of IGU will not cause additional adverse events to patients, and the occurrence of adverse events may be lower in IGU treatment over 24 weeks.

Current research shows that IGU, as a new type of anti-rheumatic drug, has good anti-inflammatory and immunosuppressive effects, and may be a potential drug for the treatment of AS in the future. The main clinical features of AS include inflammatory back pain caused by myositis and inflammation of other parts of the axial skeleton, peripheral arthritis, enteritis and anterior uveitis (30). In addition to inflammation, AS is also characterized by new bone formation in sacroiliac joints (SIJ) and the spine (31). Theories about the pathogenesis of AS include misfolding during the assembly of human leukocyte antigen (HLA)-B27, which leads to endoplasmic reticulum stress and unfolded protein response (UPR) (32). The activation of UPR gene leads to the release of TNF-α and IL-17, which is very important in the development of AS (33). The COX-2/PGE2 pathway is also important in the pathogenesis of AS (34). In addition, current evidence shows that MIF can promote inflammation and bone formation in AS (35). MIF also interacts with IL-17 and TNF-α pathways by up-regulating the expression and secretion of IL-17 and induces the production of TNF-α (35).

IGU plays an important role in suppressing immunity, inflammation, and maintaining bone balance. (1) In terms of inhibiting inflammatory factors and osteoclast intracellular signaling pathways: Bao et al. found in collagen-induced arthritis mice (CIA) that IGU can inhibit IL-17 expression while reducing TNF-α, IL-1β and IL-6 levels (36). Xu et al. confirmed that IGU can block the IL-17 pathway by targeting Act1, and IL-17 is an important cytokine involved in bone destruction in RA patients (37). The NF-κB pathway is an important intracellular conduction pathway in the process of osteoclast activation. Kohno et al. found that IGU can interfere with the translocation of NF-κB p65 into the nucleus and inhibit the activity of NF-κB (38). (2) In terms of inhibiting bone resorption: RANKL is an important signal to initiate osteoclast activation. Zhang et al. confirmed *in vitro* experiments that in mouse RAW264.7 cells, IGU can inhibit the number of osteoclasts induced by RANKL and reduce bone resorption pits (39). Guo et al. also found in bone marrow monocytes that IGU strongly inhibited RANKL-mediated osteoclastogenesis and bone resorption in a dose-dependent manner (40). IGU can also inhibit RANKL-induced osteoclast development and bone resorption in the PPARγ/c-Fos signaling pathway, and can also reduce the expression of downstream osteoclast marker genes (41). In addition, IGU not only inhibited the production of RANKL, but also significantly decreased the ratio of RANKL/OPG in serum and IL-1β-induced RA-FLSs (42). IGU inhibits the generation, differentiation, migration and bone resorption of osteoclasts induced by RANKL, and reduces the expression of nuclear activated T cell factor (NFAT) c1 and downstream osteoclast marker genes (43). These effects collectively show the effect of IGU attenuating bone erosion. Gan et al. found that IGU significantly inhibited RANKL-induced osteoclast differentiation, migration and bone resorption in RAW264.7 cells in a dose-dependent manner; the mechanism was

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TABLE 2 Summary of findings for the main comparison.

Outcomes	Illustrative comparative risks* (95% CI)		Relative effect (95% CI)	No of Participants (studies)	Quality of the evidence (GRADE)	Comments
	Assumed risk	Corresponding risk				
	Control	Primary outcomes				
Adverse events	**Study population**		**RR 0.65** (0.43 to 0.98)	542 (9 studies)	⊕⊕⊕⊖ **moderate**[1]	
	186 per 1000	**121 per 1000** (80 to 182)				
	Moderate					
	191 per 1000	**124 per 1000** (82 to 187)				
BASDAI		The mean basdai in the intervention groups was **1.62 standard deviations lower** (2.2 to 1.05 lower)		472 (8 studies)	⊕⊕⊖⊖ **low**[1,2]	SMD -1.62 (-2.2 to -1.05)
BASFI		The mean basfi in the intervention groups was **1.3 lower** (1.48 to 1.12 lower)		268 (5 studies)	⊕⊕⊖⊖ **low**[1,3]	
VAS		The mean vas in the intervention groups was **2.01 lower** (2.85 to 1.17 lower)		291 (5 studies)	⊕⊖⊖⊖ **very low**[1,2,3]	

*The basis for the assumed risk (e.g. the median control group risk across studies) is provided in footnotes. The corresponding risk (and its 95% confidence interval) is based on the assumed risk in the comparison group and the relative effect of the intervention (and its 95% CI).
CI, Confidence interval; RR, Risk ratio;
GRADE Working Group grades of evidence.
High quality: Further research is very unlikely to change our confidence in the estimate of effect.
Moderate quality: Further research is likely to have an important impact on our confidence in the estimate of effect and may change the estimate.
Low quality: Further research is very likely to have an important impact on our confidence in the estimate of effect and is likely to change the estimate.
Very low quality: We are very uncertain about the estimate.
[1] Downgraded one level due to serious risk of bias (random sequence generation, allocation concealment, blinding, incomplete outcomes) and most of the data comes from the RCTs with moderate risk of bias.
[2] Downgraded one level due to the probably substantial heterogeneity.
[3] Downgraded one level due to the total sample size fails to meet the optimal information size.

related to the activation of MAPK and NF-κB pathways (44). It shows that IGU has a direct inhibitory effect on the formation and function of osteoclasts. In addition to osteoclasts, MMPs produced by FLSs also play an important role in cartilage destruction in spondylitis (45). Du et al. treated FLS with different doses of IGU *in vitro* and then stimulated them with TNF-α, IL-1β or IL-17A. MMP-3 was significantly inhibited by 5 μg/ml IGU, but MMP-1 was significantly inhibited at 50 μg/ml. Clinical trials found that after 24 weeks of IGU (25mg, 22 times a day) treatment, the levels of MMP-1 and MMP-3 were significantly reduced (46). All these suggest that IGU prevents MMP-1 and MMP-3 from protecting cartilage (43). (3) In terms of promoting bone formation: Kohji Kuriyama et al. found that IGU can promote the differentiation of mouse bone marrow stromal cells ST2 and embryonic osteoblast precursor cells MC3T3-E1 into osteoblasts *in vitro*, and can promote BMP-2 so as to induce bone formation *in vivo* (47). In addition, Osterix is a core transcription factor that regulates bone formation and plays a key role in the differentiation of osteoblasts (48), while IGU can increase the expression of Osterix and osteocalcin (41). Song et al. also found that IGU can increase the expression of Dlx5 and Osterix and regulate the p38 pathway to promote osteoblast differentiation and maturation in mesenchymal stem cells (49). (4) In the aspect of regulating immunity: IGU can regulate immune balance by regulating T cells and related cytokine levels. Studies have shown that IGU can significantly reduce the number of Th1, Th17, follicular helper T (Tfh) cells and related transcription factors and cytokine levels, increase the number of regulatory T cells (Treg) and related transcription factors and cytokine levels (50–52). IGU also reduced the apoptosis of peripheral blood mononuclear cells, the content of IFN-γ in CD3 + T cells and the level of IL-8 in peripheral blood (53). In addition, in regulating B cells, IGU can also inhibit PKC pathway and its downstream target EGR1, thereby inhibiting B cell terminal differentiation into mature plasma cells to reduce the production of autoantibodies (54). In summary, IGU can be controlled by multiple targets, and it can inhibit cartilage and bone destruction in the pathological process of AS, and has the basis of bone protection (see Figure 14).

To promote the conclusion, the GRADE tool was utilized to rate the quality of the evidence (55). According to the GRADE handbook (56), the evidence was judged to be moderate to very low (Table 3).

The strengths of this review is that this we firstly conducted a systematic review and meta-analysis about IGU on AS. This study not only found that adding IGU to conventional therapy can improve AS, but also showed that it does not increase adverse reactions. However, the limitations is that most of the RCTs included this time did not use blinding, and did not hide the allocation of interventions, leading to a

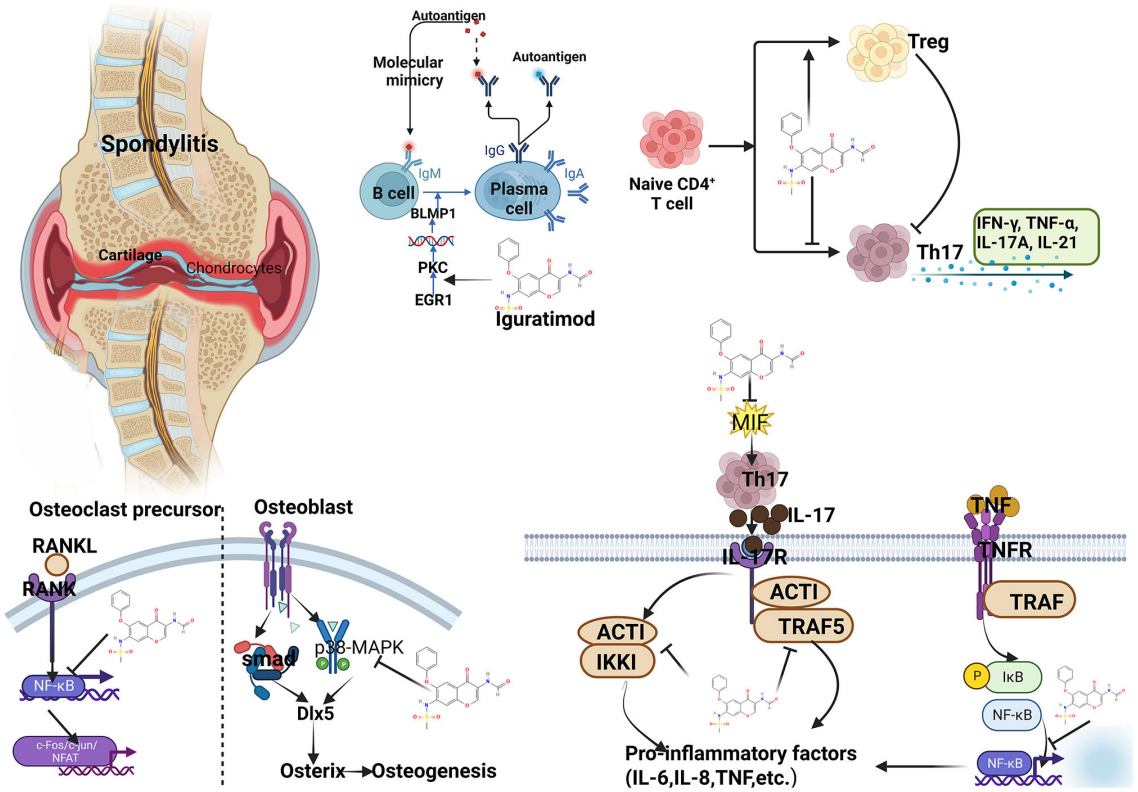

FIGURE 14
Summary of mechanism of IGU treating AS (PKC, protein kinase C; EGR1, early growth response 1; IFN-γ, interferon-γ; TNF-α, tumor necrosis factor-α; IL, interleukin; RANKL, NF-κB receptor activating factor ligand; MIF, Macrophage migration inhibitory factor; TRAF, tumor necrosis factor receptor-associated factor).

TABLE 3 Subgroup analysis results

Outcomes	Subgroup	Overall effect			Heterogeneity test		Statistical method	Studies (N)	Sample size (N)	Figure
		Effect	95%CI	P	I^2 (%)	P(Q)				
BASDAI	12 weeks	SMD=-2.28	[-4.25, -0.32]	0.023	95.47	<0.00001	Random	2	163	Figure S1
	24 weeks	SMD=-1.41	[-1.95, -0.87]	<0.00001	77.39	0.0005	Random	6	309	
VAS	12 weeks	MD=-2.01	[-3.82, -0.20]	0.03	98.84	0.00001	Random	2	165	Figure S2
	24 weeks	MD=-1.91	[-2.17, -1.64]	0	0	0.42	Random	3	126	
ESR	12 weeks	MD=-11.93	[-17.24, -6.62]	<0.0001	94.34	0.00001	Random	4	312	Figure S3
	24 weeks	MD=-9.24	[-20.13, 1.65]	0.096	59.78	0.083	Random	3	151	
CRP	12 weeks	MD=-11.41	[-17.65, -5.17]	0.0003	99.25	0.00001	Random	4	312	Figure S4
	24 weeks	MD=-8.92	[-10.40, -7.43]	0	0	0.56	Random	3	165	
TNF-α	12 weeks	MD=-5.29	[-7.93, -2.64]	0.00009	96.66	0.00001	Random	2	147	Figure S5
	24 weeks	MD=-7.22	[-7.94, -6.51]	0	0	0.98	Random	2	110	
Adverse events	12 weeks	RR=0.78	[0.43, 1.41]	0.42	26.82	0.25	Fixed	4	293	Figure S6
	24 weeks	RR=0.55	[0.31, 0.97]	0.038	0	0.44	Fixed	5	249	

high risk of bias in the results. The number of RCTs included in this study is small, and the number of participants involved is not more than 1,000, which may affect the accuracy of the results. Moreover, most of the patients included in the study included this time are Chinese, which may affect the applicability of the results. Therefore, high-quality RCTs involving more countries and regions are needed in the future to revise or verify the results of this meta-analysis.

5 Conclusion

Through the systematic evaluation and meta-analysis of this study, it can be clarified that IGU as a new multi-targeted DMARD may have multiple benefits in the treatment of AS.

Data availability statement

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

Author contributions

LZ, YiD, KY, HC are responsible for the study concept and design. LZ, YiD, QH, ZL, KY, WH, YuD, JF, HC are responsible for the data collection, data analysis and interpretation. LZ and KY drafted the paper. HC supervised the study. All authors contributed to the article and approved the submitted version.

Conflict of interest

Author JF was employed by company ZCCC Jinzhu Transportation Construction Co. Ltd.

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

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Supplementary material

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Epigenetic dysregulation of autophagy in sepsis-induced acute kidney injury: the underlying mechanisms for renoprotection

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Sepsis-induced acute kidney injury (SI-AKI), a common critically ill, represents one of the leading causes of global death. Emerging evidence reveals autophagy as a pivotal modulator of SI-AKI. Autophagy affects the cellular processes of renal lesions, including cell death, inflammation, and immune responses. Herein, we conducted a systematic and comprehensive review on the topic of the proposed roles of autophagy in SI-AKI. Forty-one relevant studies were finally included and further summarized and analyzed. This review revealed that a majority of included studies (24/41, 58.5%) showed an elevation of the autophagy level during SI-AKI, while 22% and 19.5% of the included studies reported an inhibition and an elevation at the early stage but a declination of renal autophagy in SI-AKI, respectively. Multiple intracellular signaling molecules and pathways targeting autophagy (e.g. mTOR, non-coding RNA, Sirtuins family, mitophagy, AMPK, ROS, NF-Kb, and Parkin) involved in the process of SI-AKI, exerting multiple biological effects on the kidney. Multiple treatment modalities (e.g. small molecule inhibitors, temsirolimus, rapamycin, polydatin, ascorbate, recombinant human erythropoietin, stem cells, Procyanidin B2, and dexmedetomidine) have been found to improve renal function, which may be attributed to the elevation of the autophagy level in SI-AKI. Though the exact roles of autophagy in SI-AKI have not been well elucidated, it may be implicated in preventing SI-AKI through various molecular pathways. Targeting the autophagy-associated proteins and pathways may hint towards a new prospective in the treatment of critically ill patients with SI-AKI, but more preclinical studies are still warranted to validate this hypothesis.

KEYWORDS

sepsis, acute kidney injury, autophagy, protection, mechanism

Introduction

Sepsis, one of the common diseases in the intensive care units, seriously threatens the lives of sufferers, contributing to 30-50% of deaths in hospitals (1). As a result of the dysregulated host response to infection, severe systemic inflammation may induce septic shock, disseminated intravascular coagulation (DIC), and progressive multi-organ dysfunction syndrome (MODS) (2). The commonly affected organs include the heart, lungs, liver, brain, intestine, and kidneys. During sepsis, activation of the sympathetic nervous system, the release of vasoactive substances, and endothelial injury, together contribute to the redistribution of blood flow and microcirculation disturbances (3). These factors remarkably damage the kidney tissue (e.g. renal tubular) and therefore induce acute kidney injury (AKI) and even acute renal failure (ARF). ARF was found to be the most common complication of sepsis, accounting for nearly 50% of the incidence rate (4). On the other hand, it was reported that approximately 76% of in-hospital deaths are caused by sepsis-induced AKI (SI-AKI) (5). Since multidrug-resistant bacteria and adverse events are common in sepsis, septic ARF has a significantly higher mortality rate than that of non-sepsis-related ARF (caused by other pathogenic factors or some diseases) (6). To effectively prevent and treat septic AKI or ARF, intensive efforts have been made to develop innovative therapeutic measures and explore the detailed molecular pathological mechanisms underlying SI-AKI. However, due to the complex character of SI-AKI, the exact pathogenesis mechanisms for septic AKI are not completely addressed, prohibiting or arresting effective treatments for septic AKI.

At present, there is growing evidence that autophagy plays a role in the pathogenesis process of SI-AKI (7). Autophagy is an adaptive catabolic process and is commonly correlated to cellular death, protection, or survival. It conserves the degradation of eukaryotic cells and the recycling process, maintaining cellular homeostasis by engulfing cellular targets (i.e., pathogens, unfolded proteins, carbohydrates, lipids, nucleic acids, and damaged organelles) (8). Autophagy can be regulated by a complex signaling network comprised of autophagy-related genes (ATC). Mitophagy and lysophagy are the common types of selective autophagy. Autophagy is considered to be a cellular stress triggered by a multiplicity of adverse environmental cues, i.e., hypoxia, oxidative stress, and nutrient depletion (9). Experimental and clinical findings reveal that autophagy may serve as a pathogenic mediator of human diseases by regulating inflammation, innate immunity, and host defense. Dysregulated or maladaptive autophagy with propathogenic responses was found to be associated with the pathogenesis of disorders (10).

For the kidneys to function normally, autophagy is necessary (11). Besides, the presence of kidney pathologies in mice bearing genetic deletions of key autophagy regulator proteins. For example, in an animal model with targeted deletions of *Atg5* or *Atg7*, chronic kidney disease could be induced (12). Therefore, autophagy may act as a key safeguard against the declination of kidney function. Also, it was reported that autophagy generally protects the kidney from various injuries, e.g. sepsis, renal ischemia-reperfusion (I/R), or exposure to nephrotoxins (13). However, the exact molecular mechanisms underlying the action of autophagy in AKI are still exploring.

In this review, we focused specifically on the roles of autophagy in sepsis-induced AKI according to the current evidence. Elucidating the biological effects of autophagy in septic AKI is of pivotal importance, which may provide strategies and targets for therapeutic interventions in clinical practice.

A search of the literature

To identify the studies that reported the association between autophagy and sepsis-induced AKI, we conducted a comprehensive literature review in the common-used databases, i.e., MEDLINE, Web of Science, Google Scholar, EMBASE, and Cochrane Library. The reference list in the review or original studies was also retrieved to identify additional relevant studies. Data from relevant studies were extracted using a data collection table. The following information was noted, including the first author of the included studies, year of publication, experimental model or participant, methods for establishing sepsis-induced AKI, the status of autophagy, associated genes or pathways in the action of autophagy, and the main findings of the relevant studies. Finally, there were 41 experimental and clinical studies (7, 14–33) (34–53) included in the review. The selection process for screening the relevant studies shown in Supplementary Figure 1. For the methods for establishing the SI-AKI, cecal ligation and puncture (CLP) were used in *in-vivo* studies, while lipopolysaccharide (LPS) was applied in *in vitro* studies.

Different autophagy levels in septic AKI

Among the 41 relevant studies, 24 of them (24/41, 58.5%) reported that the autophagy status was activated in septic AKI, while eight eligible studies showed that autophagy inhibition occurred in the kidney under sepsis and the remaining nine studies reported that autophagy elevated in early stage but declined in the later phase during sepsis-induced AKI.

Autophagy, a form of programmed cell death different from apoptosis, occurs in all eukaryotic cells and is associated with cellular turnover and energy balance. Death of cells occurs when apoptosis appears, whereas autophagy is a "double-edged sword" for both survival and death (54, 55). Apoptosis and autophagy are interconnected in some way. To protect cells from apoptosis and necrosis under stress, autophagy is commonly activated. Nevertheless, excessive autophagy can increase apoptosis due to mitochondrial damage. Autophagy plays a protective role in proximal tubular cells of the kidney against AKI (11). Of note, autophagy is considered to play a double role during sepsis. Basal autophagy functions the protection of the injuried kidney by eliminating toxic oxidative proteins. However, severe stress (i.e., ROS eruption) may induce excessive autophagy, leading to autophagic cell death (56). It is reported that autophagy is initiated early after sepsis, protecting against endotoxic kidney damage (57). Afterward, autophagic cell death may cause a phase of dysfunction, aggravating the sepsis-induced oxidative injury.

At present, specific molecular mechanisms underlying sepsis-associated AKI are not fully understood. Several potential mechanisms could be involved (58). First, autophagy can protect proximal tubular cells from mitochondrial oxidative stress and ischemic injury. 'Mitophagy' is a specific type of autophagy, which is characterized by the clearance of mitochondria by autophagy. Second, autophagy also plays role in protecting proximal tubular cells from DNA damage. Third, autophagy can reduce the abnormal protein accumulation of proximal tubular cells.

Since the level of autophagy flux was different among the 41 included studies, exploring the underlying mechanisms of autophagy is of great interest and has important implications for septic AKI research. Here, we summarize recent laboratory and clinical studies, focusing on critical factors in the pathophysiology of sepsis-associated AKI: microcirculatory dysfunction, inflammation, NOD-like receptor protein 3 inflammasome, microRNAs, extracellular vesicles, autophagy and efferocytosis, inflammatory reflex pathway, vitamin D, and metabolic reprogramming. Lastly, identifying these molecular targets and defining clinical subphenotypes will permit precision approaches in the prevention and treatment of SI-AKI.

Autophagy activation in septic AKI

It is possible that autophagy plays a key role in the recovery of AKI by promoting cell survival. Autophagy can be triggered in response to various types of stress, including sepsis. A critical cytoprotective role for autophagy in sepsis-mediated AKI has been found in recent years (11). The protective effects exerted by autophagy may be associated with the removal of damaged mitochondria or mitophagy, which predominantly affects the mitochondria-rich proximal tubule cells (58). Currently, evidence that harnessing the autophagic machinery on SI-AKI is still controversial. The levels of autophagy flux during SI-AKI are different among studies.

Twenty-four publications reported the autophagy level was activated during sepsis-induced AKI. Alexander et al. (27) performed the autopsy on 17 patients who died from coronavirus disease 2019 (COVID-19) and molecular characteristics were compared with archived cases of S-AKI and non-sepsis causes of AKI. They found that the autophagy level was significantly higher in sepsis-induced AKI than in non-sepsis-related AKI (P=0.023). However, the autophagy status was comparable between sepsis AKI and COVID AKI (P=0.621). This clinical trial indicated that mitochondrial autophagy dysfunction might play a pivotal role in SI-AKI, which might provide novel diagnostic and therapeutic targets for treating SI-AKI. In another clinical study developed by Feng et al. (23), the investigators also revealed the autophagy status was elevated in SI-AKI patients. As a result, exploring the molecular mechanisms underlying the actions of autophagy dysfunction in septic AKI may be clinically instructive. Currently, 24 experimental studies had investigated the association between autophagy and SI-AKI. Nevertheless, the detailed pathomechanisms of autophagy contributed to the pathogenesis of septic AKI.

The characteristics and the main findings of the 24 included studies were summarized in Table 1. Figure 1 (left column) displayed the molecular mechanisms of activated autophagy during SI-AKI.

mTOR signaling pathway

The mammalian target of the rapamycin (mTOR) pathway is one of the pivotal signaling pathways with critical biological function in multiple diseases (59), including SI-AKI (60). mTOR has been reported to play a central role in the regulation of autophagy, characterized by inhibiting autophagy in the biological process of growth factors and abundant nutrients (61). The calcium/calmodulin-dependent protein kinases (CaMK) were found to regulate septic inflammation. Zhang et al. (14) demonstrated that CaMKIV signaling mediated the autophagic response to LPS-associated septic AKI by inhibiting GSK3β and FBXW7 expression and maintaining mTOR levels. mTOR and AMP-activated protein kinase (AMPK) have been reported to correlate with in the development of autophagy in AKI (62). BECN1, Bcl-2, and LC3-II are pivotal autophagy-related proteins. Increased LC3-II and BECN1 expression have been found in SI-AKI, indicating the autophagy status enhanced during sepsis. Zhao et al. (18) showed that elevated autophagy occurred in SI-AKI, while a high level of SIRT3 could protect against AKI by modulating AMPK/mTOR-mediated autophagy. In a mouse model of SI-AKI conducted by CLP, Sang et al. (32) also confirmed that the kidney autophagy level was elevated. They further found that PTEN/AKT/mTOR signaling pathway was involved in this action. The above studies indicated that mTOR was one of the key targets for the activation of autophagy during SI-AKI.

non-coding RNA

Both long non-coding RNAs (lncRNAs) and microRNA (miRNA) have been suggested to play essential roles in various diseases, including SI-AKI (63, 64). It is known that lncRNA regulates the activities of miRNA through the lncRNA-miRNA interactions. On the topic of the present study, we also found that both lncRNA and miRNA participated in the development of autophagy-mediated SI-AKI. PlncRNA-1 was reported to initiate malignancy in multiple cancers and play roles in inflammatory diseases (65). Fu et al. (16) showed that renal autophagy was activated in SI-AKI by up-regulating LC3-II and down-regulating PlncRNA-1 and BCL2 levels, while overexpression of PlncRNA-1 inhibited autophagy by up-regulating BCL2 expression. Enhanced autophagy was also observed in Feng et al.' study (23). Upregulated NEAT1 but downregulated miR-22-3p was found in patients with SI-AKI and in LPS-induced HK-2 cells. Liu et al. (23) suggested that ATG7 promoted autophagy (characterized by increasing levels of Beclin-1, LC3-I, and LC3-II) in SI-AKI, which could be inhibited by miR−526b. In a recent study conducted by Han et al. (30), the authors demonstrated that kidney autophagy was activated, while LncRNA NKILA silencing could protect HK-2 cells from SI-AKI by decreasing CLDN2 by sponging miR-140-5p. Sang et al. (32) reported that an increased level of LC3-II was found in CLP-

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TABLE 1 The characteristics and the main findings of the 24 relevant studies reporting activation of autophagy in SI-AKI.

Study/ Reference	Experimental model/ participant	Methods for establishing sepsis-induced AKI	Status of autophagy	Associated genes or pathways	Main findings
Zhang et al. (14)	Mice	LPS	Augment	Down-regulating GSK3β and FBXW7; Maintaining mTOR expression	CaMKIV signaling mediated the autophagic response to sepsis-induced AKI, by inhibiting GSK3β and FBXW7 expression and maintaining mTOR.
Chen et al. (15)	Mice and HK-2 cells	CLP, LPS	Activated 1 day after CLP	Increasing LC3-II and Rap expression, inhibiting 3-MA protein	Autophagy activation was observed after CLP, while the protective effect developed by Klotho in sepsis-induced AKI might be irrelevant to autophagy.
Fu et al. (16)	NRK-52E cells	LPS	Activated	Up-regulating LC3-II and down-regulating PlncRNA-1 and BCL2	Overexpression of PlncRNA-1 inhibited autophagy by up-regulating BCL2 expression in septic AKI.
Zhao, et al. (18)	Mice	CLP	Activated	Increased LC3-II and BECN1, decreased expression levels of SIRT3	High level of SIRT3 protects against sepsis-induced AKI by modulating AMPK/mTOR-mediated autophagy.
Jia et al. (19)	Rat	CLP	Activated	The expression of LC3II, Atg5, and beclin 1 were significantly increased	Alpha-Lipoic Acid improved the renal functioning in septic AKI by upregulating Atg5, Atg7, and beclin-1 expression, but decreased p62 levels in the kidney.
Wu et al. (20)	Mice	CLP	Activated	Beclin1 and LC3−II/I were significantly elevated	The activation of autophagy might aggravate the renal injury in mice. It was speculated that inhibiting autophagy might increase the survival rate of patients with septic AKI.
Zhang et al. (21)	Mice and HK-2 cells	LPS	Activated	SIRT6 and LC3B-II/LC3B-I expression were significantly increased	Activation of autophagy and increased inflammation were observed in LPS-induced septic AKI. Overexpression of SIRT6 induced autophagy of HK-2 cells.
Zheng et al. (22)	HK-2 cells	LPS	Activated	Increased protein expression levels of beclin-1	Downregulation of ATM significantly inhibited autophagy and inflammatory response in LPS-induced AKI.
Feng et al. (23)	Patients and HK-2 cells	LPS	Activated	Increased level of Beclin-1, LC3-II/I, and NEAT1	Upregulated NEAT1 but downregulated miR-22-3p was observed in patients with sepsis and in LPS-induced HK-2 cells.
Gao et al. (24)	Mice	CLP	Activated	Increased the level of LC3-II but decreased the expression of p62	Polydatin protected against mitochondrial dysfunction in sepsis-induced-AKI by activating mitophagy *via* upregulating SIRT1.
Liu et al. (25)	Mice and HK-2 cells	CLP and LPS	Activated	Increased expression of Beclin-1, LC3-I, LC3-II, and ATG7	ATG7 promoted autophagy in sepsis–induced AKI and was inhibited by miR−526b
Miao et al. (26)	Mice	LPS	Activated	Enhanced LC3-II and 15-PGDH protein expression	Blockade of 15-PGDH promoted autophagic response, alleviating LPS-induced septic AKI.
Alexander et al. (27)	Patients (n=17)	Gene expression analysis	Enhanced	NA	The autophagy level was significantly higher in sepsis AKI than nonsepsis-related AKI (P=0.023), while it was comparable between sepsis AKI and COVID AKI (P=0.621).
Chen et al. (28)	Mice and HK-2 cells	LPS	Activated	Activation of TLR4	Ascorbate protected against LPS-induced AKI by enhancing mitophagy mediated by PINK1-PARK2 axis.
Guo et al. (29)	Rat, HK-2, RTECs cells	CLP and LPS	Activated	Increased LC3II, and decreased level of p62	BMSCs protected rats against sepsis-induced AKI by promoting mitophagy *via* upregulating SIRT1/Parkin.
Han et al. (30)	HK-2 cells	LPS	Activated	Increased LC3II and Beclin1	LncRNA NKILA silencing protected HK-2 cells from sepsis-mediated AKI by decreasing CLDN2 through sponging miR-140-5p.
Li et al. (7)	Mice	LPS	Activated	Increased LC3II and RIP3	RIP3 suppressed autophagic degradation *via* impeding the transcription factor EB -lysosome pathway and the nuclear translocation in septic AKI.

(Continued)

TABLE 1 Continued

Study/ Reference	Experimental model/ participant	Methods for establishing sepsis-induced AKI	Status of autophagy	Associated genes or pathways	Main findings
Pan et al. (31)	HK-2 cells	LPS	Activated	Beclin1, ATG5, and LC3B-II was increased; p62 expression was downregulated	Inhibition of TREM-1 increased autophagy in LPS-induced cell model by activating the NF-κB pathway (P-p65, p65, P-IκBα, and IκBα).
Sang et al. (32)	Mice	CLP	Activated	Elevated LC3 II/I and the reduction of p62	Mir-214 protected against sepsis-induced AKI by decreasing oxidative stress and suppressing autophagy *via* regulation of the PTEN/AKT/mTOR pathway.
Tan et al. (33)	Mice	CLP	Activated	The increase of the ratio of LC3–II/I and decrease of the expression of p62	The protective effect of inhibition of aerobic glycolysis against sepsis–induced AKI might be associated with the induction of autophagy *via* the lactate/SIRT3/AMPK pathway.
Wang et al. (34)	Mice	CLP and LPS	Activated	The levels of LC3-II were increased and peaked at 24 h; TOM20 and TIM23 were reduced	Mitophagy was activated in renal tubular cells during septic AKI by up-regulating the autophagy adaptor optineurin (OPTN) expression, which was mediated by the PINK1-PARK2 pathway.
Li et al. (35)	Mice and HK-2 cells	LPS	Activated	The ratio of LC3B–II/LC3B –I increased and the level of p62 decreased	Sodium hydrosulfide hydrate (NaHS) prevented sepsis-associated AKI by promoting autophagy to suppress renal tubular epithelial cell apoptosis and reduce inflammatory factors.
Li et al. (36)	Mice	LPS	Activated	Increased LC3BII expression in platelets	LPS induced platelet autophagy by generating mitochondrial ROS. TLR4 inhibitor TAK242 might effectively alleviate septic AKI by inhibiting platelet GPIIb/IIIa, and reducing platelet activation.
Li et al. (37)	Rat	CLP	Activated	Increased the expression of LC3II	Ulinastatin protected the adhesion junction and ameliorated the perfusion of kidney capillaries during sepsis by the inhibition of autophagy and the up-regulation of VE-cadherin expression.

Atg7, autophagy-related gene 7; LC3, microtubule-associated protein 1 light chain 3; CLP, Cecal ligation and puncture; LPS, Lipopolysaccharide; mTOR, mechanistic target of rapamycin; Pink1, PTEN induced putative kinase 1; Parkin, Parkin RBR E3 ubiquitin protein ligase; NLRP3, NLR family pyrin domain containing 3; HK-2, Human kidney proximal tubular epithelial (HK-2) cells; BMSCs, Bone marrow-derived mesenchymal stem cells; RTECs, Renal tubular epithelial cells; RIP3, Receptor interacting protein kinase 3; ATM, ataxia-telangiectasia mutated; 15-PGDH, 15-hydroxyprostaglandin dehydrogenase; TREM-1, Triggering receptor expressed by myeloid cells; TLR4, Toll Like Receptor 4.

induced SI-AKI, indicating the autophagic level was active. They also observed that miR-214 protected against sepsis-induced AKI by decreasing oxidative stress and suppressing autophagy by regulating the PTEN/AKT/mTOR pathway. These data collectively implied that the enhanced kidney autophagy occurred in SI-AKI, which was partially mediated by several specific non-coding RNAs, e.g. PlncRNA-1, miR-22-3p, miR–526b, LncRNA NKILA, miR-140-5p, and miR-214.

Mitophagy

Mitophagy, an evolutionarily conserved biological process, is one of the pivotal cytoprotective mechanisms. It functions to remove the damaged mitochondria and maintain a healthy mitochondrial population (66). Gao et al. (24) reported that polydatin could protect against mitochondrial dysfunction in SI-AKI by activating Parkin-dependent mitophagy *via* upregulating SIRT1 and inhibiting NLRP3 activation. Ascorbate is a precursor for carnitine and catecholamine synthesis. It protects against oxidative stress in various diseases (67). Recently, Chen et al. (28) showed that ascorbate protected against LPS-induced AKI by enhancing mitophagy mediated by the PINK1-PARK2 axis. Bone marrow-derived mesenchymal stem cells (BMSCs) play roles in self-renewal and multi-differentiation, functioning in tissue repair and regeneration (68). It was reported that BMSCs protected rats against SI-AKI by promoting mitophagy *via* upregulating SIRT1/Parkin (29). In line with the above studies, Wang et al. (34) also confirmed that autophagy was increased (characterized by a high level of LC3-II) during SI-AKI. They found that mitophagy was activated in renal tubular cells in SI-AKI by up-regulating the autophagy adaptor optineurin (OPTN) expression, which was affected by the PINK1-PARK2 pathway. The above studies demonstrated that several substances (i.e., polydatin, ascorbate, and BMSCs) significantly protected against SI-AKI, which might partially mediate by the activation of mitophagy.

Sirtuins

Sirtuins belong to the family of NAD^+-dependent histone deacetylases, which play roles in energy metabolism, inflammation, and tumorigenesis (69). According to some included studies, Sirtuins (e.g. SIRT3, SIRT6, and SIRT1) have

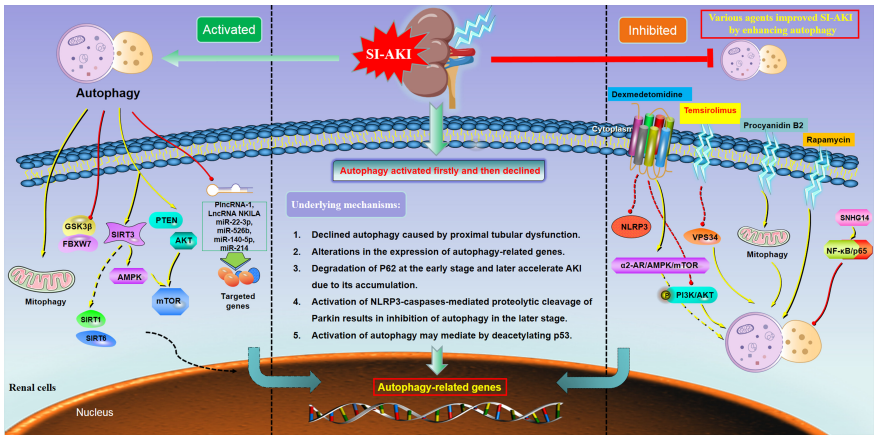

FIGURE 1
Main molecular mechanisms underlying the dual role of autophagy in sepsis-induced acute kidney injury. Under the condition of SI-AKI, the autophagy level presented with three tendencies, including activation (left column), activated first and then declined (middle column), and inhibition (right column). These distinct patterns of autophagy in SI-AKI might be regulated by multiple associated genes and a series of downstream signaling. Abbreviation: mTOR= mechanistic target of rapamycin; NLRP3, NLR family pyrin domain containing 3; Sirt1, Sirtuin 1; Sirt3, Sirtuin 3; Sirt6, Sirtuin 6; GSK3β, Glycogen Synthase Kinase 3β; AMPK, Adenosine 5′-monophosphate-activated protein kinase; PI3K, Phosphatidylinositol 3-kinase; VPS34, Vacuolar protein sorting 34; PTEN, Phosphatase and tensin homolog.

been implicated in involving in the activation of autophagy during SI-AKI. Upregulation of SIRT3 has been reported to protect against sepsis-induced AKI (18). Similarly, the protective effects of inhibition of aerobic glycolysis against SI-AKI were found to be associated with the induction of autophagy, which might be partly due to the upregulation of the SIRT3/AMPK pathway (33). In Zhang et al.'s study (21), activation of autophagy (increased LC3B-II/LC3B-I expression) and increased inflammation were observed in LPS-induced septic AKI. The authors further indicated that overexpression of SIRT6 might induce autophagy of HK-2 cells (21). Gao et al. (24) showed that polydatin could protect against mitochondrial dysfunction in SI-AKI by upregulating the expression of SIRT1. These studies suggest that Sirtuins are important in autophagic changes during SI-AKI and are the potential therapeutic targets of SI-AKI.

Other potential mechanisms

In addition to the above molecular molecules and pathways, the roles of autophagic activation in SI-AKI might also cause or mediate by some other biological agents. Chen et al. (15) showed that SI-AKI was accompanied by increasing LC3-II and Rap expression but inhibiting 3-MA protein. They observed that autophagy activation presented after CLP, while the protective effect developed by Klotho in SI-AKI might be irrelevant to autophagy. Jia et al. (19) reported that Alpha-Lipoic Acid could improve renal functioning in SI-AKI by upregulating the expression of autophagy-associated genes, such as Atg5, Atg7, and beclin-1. It was suggested that Beclin1 and LC3–II/I were significantly increased in SI-AKI, while the activation of autophagy might aggravate the renal injury (20). Zheng et al. (22) also confirmed that autophagy was enhanced in SI-AKI. They next found that downregulation of ataxia-telangiectasia mutated (ATM) significantly suppressed autophagy and inflammatory response in LPS-induced AKI. 15-hydroxyprostaglandin dehydrogenase (15-PGDH) is an important enzyme in the degradation of prostaglandins. Miao et al. (26) demonstrated that the blockade of 15-PGDH promoted autophagic response, alleviating LPS-induced SI-AKI. Receptor interacting-protein kinase 3 (RIP3) has been found to function as protect against renal tubular injury and renal dysfunction during septic AKI. Li et al. (7) revealed that RIP3 suppressed autophagic degradation by impeding the transcription factor EB-lysosome pathway and the nuclear translocation in SI-AKI.

Triggering receptor expressed by myeloid cells (TREM-1) is an amplifier of inflammatory responses induced by infections. Pan et al. demonstrated that inhibition of TREM-1 elevated autophagy in SI-AKI by activating the NF-κB pathway (P-p65, p65, P-IκBα, and IκBα). Sodium hydrosulfide hydrate (NaHS) has been implicated in preventing SI-AKI by promoting autophagy to suppress renal tubular epithelial cell apoptosis and reduce inflammatory factors (35). LPS can increase the production of intracellular ROS *via* Toll-like Receptor 4 (TLR4), which can lead to mitochondrial damage and activate platelets. LPS-induced platelet autophagy by generating mitochondrial ROS. Li et al. (36) showed that TLR4 inhibitor TAK242 might effectively alleviate SI-AKI by inhibiting platelet GPIIb/IIIa and platelet activation. Ulinastatin, a urinary trypsin inhibitor, functions to control a series of proinflammatory mediators and cytokines. A recent study showed that Ulinastatin protected the adhesion junction and ameliorated the perfusion of kidney capillaries during SI-AKI by suppressing autophagy and elevating VE-cadherin expression (37). Taken together, all the above-mentioned genes and substances might be involved in the biogenesis and biological functions of autophagy activation in SI-AKI. Targeting these affected proteins may be one of the effective therapeutic regimens that can protect against SI-AKI.

In summary, the above 24 included studies demonstrated that autophagy was increased during SI-AKI, while some drugs, substances, or molecules exert protective effects against SI-AKI

also due to their properties on the promotion and enhancement of autophagy. One possible mechanism is that increased autophagy may be one of the phenotypes of the early stage of SI-AKI, which can be considered as a physiological compensatory response. Nevertheless, some specific external interventions applied for enhancing the autophagy flux may contribute to the renoprotective effects on SI-AKI, which may be partially related to a high level of autophagy in the later stage of SI-AKI may promote renal cell survival.

Autophagic inhibition in SI-AKI

Among the 41 included studies, a majority of them indicated that the autophagy flux was increased in SI-AKI, while there were eight studies (8/41, 19.5%) suggested that autophagy was inhibited during SI-AKI (Table 2). These experimental animal and cellular models indicated that autophagy was diminished in the kidney of SI-AKI and that proximal tubule cells fail to promote autophagy. Since the inhibition of autophagy was observed in SI-AKI, enhanced autophagy flux may effectively improve renal recovery.

Howell et al. (38) found that diminished autophagy was associated with renal dysfunction during SI-AKI. Meanwhile, the authors also observed that VPS34 expression was inhibited and the mTOR was activated. Further, they discovered that SI-AKI animals treated with temsirolimus (an mTOR inhibitor) or inhibiting VPS34 expression significantly improve renal function by elevating autophagy. Yang et al. (53) reported that the autophagy level was inhibited during SI-AKI, which was characterized by decreased LC3-II/LC3-I ratio and enhanced p62 expression. In this study, SNHG14 inhibited cell autophagy and promoted inflammatory cytokine production in SI-AKI. SNHG14/miR-495-3p/HIPK1 interaction network plays a key role in the septic process, which might be modulated *via* the NF-κB/p65 signaling. Rapamycin, an inducer of autophagy, has been found to reduce the extent of SI-AKI. Sunahara et al. (48) reported that the number of autophagosomes decreased at 24h after CLP, indicating that autophagy was restrained during SI-AKI. They next found that rapamycin could improve renal functioning by accelerating autophagy. Li et al. (40) showed that the levels of LC3II were reduced in SI-AKI compared to the controls, but the expression of p62 was increased. The authors revealed that recombinant human erythropoietin (rhEPO) could alleviate SI-AKI by activating autophagy through AMPK/SIRT1 pathway. Feng et al. (50) reported that treatment with human umbilical cord blood mononuclear cells (hUCBMNCs) protected against LPS-induced AKI by increasing autophagy in the kidney. The underlying mechanisms might be associated with the decreased expression of several proteins (e.g. mfn2, PINK1, Parkin, and LC3-II) and the elevated expression of Drp1 and p62, which might be partly mediated by inhibiting Nrf2. A more recent study developed by Yu et al. (45) showed that NF-κB inhibitor 270 could protect against SI-AKI by promoting autophagy by reducing inflammation responses, which might be associated with the inhibition of NF-κB transcriptional activity, NF-κB, and JNK signaling pathways.

Mitophagy also plays a key role in the inhibition of autophagy in SI-AKI. Procyanidin B2 (PB2), one of the common antioxidants, exerts excellent anti-oxidative and anti-inflammatory effects on multiple diseases (70). Decreased LC3, Pink1, and Parkin, while increased TOM20 and TIM23 were identified in Liu et al.'s study (42). The authors further suggested that the protective effects of antioxidant Procyanidin B2 on mitochondrial dynamics in SI-AKI might be partially through the elevation of mitophagy level, which might be associated with the increased nuclear translocation of Nrf2.

Dexmedetomidine (DEX), a selective α2-adrenoreceptor agonist, functions with the effects of the sedative, analgesic, and anti-anxiety (71). Besides, mounting experimental studies demonstrated that DEX has outstanding antioxidant, anti-apoptosis, and anti-inflammatory effects (72). Yang et al. (43) reported that the autophagy level was decreased in SI-AKI (characterized by decreased expression of LC3-II and Beclin-1). The investigators subsequently found that DEX protected against LPS-induced AKI by enhancing autophagy, which might be correlated to the inhibition of NLRP3 inflammasome and the activation of the α2-AR/AMPK/mTOR pathway. Consistent with Yang et al.' study, Zhao also observed a reduced autophagy flux during SI-AKI (characterized by decreased Beclin-1 and LC3 II expression). They showed that DEX ameliorated LPS-induced AKI by promoting autophagy by inhibiting the phosphorylation levels of PI3K, AKT, and mTOR.

Taken together, the above eight included studies demonstrated that the autophagy level was decreased during SI-AKI, while the promotion of autophagy flux might significantly improve the renal function, which suggested that autophagy played a protective role against SI-AKI. The mechanisms of autophagy inhibition in SI-AKI and the potential molecular mechanisms were illustrated in Figure 1 (right column).

Autophagy rises first and then falls during SI-AKI

Within the topic of this study, nine included studies demonstrated that autophagy rose firstly and decreased later in SI-AKI (Table 3). Besides, some eligible studies also showed that the autophagy level increased early, then declined, and increased again later. Autophagy is commonly upregulated by environmental stress, such as inflammatory mediators, mitochondrial dysfunction, and ATP depletion, to maintain homeostasis (73). In addition, the autophagy process is a tightly regulated machinery, which can remove damaged proteins and organelles (74). According to the available data from the included studies in this study, the early stage of SI-AKI could be defined as less than 8h after CLP or LPS treatment, while sepsis >8h could be thought as the late stage of SI-AKI. Hsiao et al. (46) observed that the expression of LC3-II increased at 3h and 6 h after CLP but sequentially decreased to the basal level at 9h and 18 h after CLP. In response to septic insult, the level of autophagy transiently elevated in kidney tissue at CLP_{3h}. Due to renal dysfunction and morphological injury, renal autophagy declined at late sepsis, which contributed to proximal tubular dysfunction in an animal model of SI-AKI. *In vitro* study, siRNA knockdown of Atg7 on NRK-52E cells significantly declined the level of LC3-II. This is the first *in vivo* study to detect the decline

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TABLE 2 The characteristics and the main findings of the 9 relevant studies reporting inhibition of autophagy in SI-AKI.

Study/ Reference	Experimental model/ participant	Methods for establishing sepsis-induced AKI	Status of autophagy	Associated genes or pathways	Main findings
Howell et al. (38)	Mice	CLP; LPS administration	Inhibited	Inhibition of LC3b and VPS34 expression and activation of mTOR	During sepsis, diminished autophagy was associated with renal dysfunction, while treatment with temsirolimus or inhibiting VPS34 expression significantly improve renal function by elevating autophagy.
Sunahara, et al. (48)	Mice	CLP	Inhibited	The number of autophagosomes decreased at 24h after CLP	Autophagy significantly reduced in the kidney during the acute phase of sepsis. Rapamycin could improve the renal functioning by accelerating autophagy.
Li et al. (40)	HK-2 and HEK-293 cells	LPS	Inhibited	Decreasing the levels of LC3II, but increasing the p62 expression through the AMPK/SIRT1 pathway	Recombinant human erythropoietin (rhEPO) alleviated septic AKI by activating autophagy through AMPK/ SIRT1 pathway.
Feng et al. (23)	Rat	LPS	Inhibited	Decreased expression of mfn2, PINK1, Parkin, and LC3-II, while increased the level of Drp1 and p62, which mediated by inhibiting Nrf2	Treatment with human umbilical cord blood mononuclear cells (hUCBMNCs) protected against LPS-induced AKI by increasing autophagy in kidney.
Liu et al. (25)	Mice	LPS	Mitophagy was decreased	The levels of LC3, Pink1, and Parkin were decreased, while the expression of TOM20 and TIM23 were increased	The protective effects of antioxidant Procyanidin B2 on mitochondrial dynamics in septic AKI might partially through the elevation of mitophagy level, which might be associated with the increased nuclear translocation of Nrf2.
Yang et al. (43)	Rat	LPS	Inhibited	Decreased expression of LC3-II, beclin-1, and NLRP3, but increased expression of p62	Dexmedetomidine protected against LPS-induced AKI by enhancing autophagy *via* inhibiting NLRP3 inflammasome and activating α2-AR/AMPK/mTOR pathway.
Zhao et al. (44)	Rat	LPS	Inhibited	Decreased the expression of Beclin-1, LC3 II, and PINK1	Dexmedetomidine ameliorated LPS-induced AKI by enhancing autophagy through inhibiting the phosphorylation levels of PI3K, AKT, and mTOR.
Yang et al. (53)	HK-2 cells	LPS	Inhibited	Decreased LC3-II/LC3-I ratio and enhanced p62 expression	SNHG14 inhibited cell autophagy and promoted inflammatory cytokine production in a SI-AKI cell model. SNHG14/miR-495-3p/HIPK1 interaction network played role in this action *via* modulating NF-κB/p65 signaling.
Yu et al. (45)	Mice	LPS	Inhibited	Elevated the protein expression of LC3A and p62	NF-κB inhibitor 270 protected against septic AKI by promoting autophagy *via* the inhibition of NF-κB transcriptional activity, NF-κB, and JNK signaling pathways mediated inflammation responses.

LC3, microtubule-associated protein 1 light chain 3; CLP, Cecal ligation and puncture; LPS, Lipopolysaccharide; mTOR, mechanistic target of rapamycin; Pink1, PTEN induced putative kinase 1; Parkin, Parkin RBR E3 ubiquitin protein ligase; NLRP3, NLR family pyrin domain containing 3; HK-2, Human kidney proximal tubular epithelial (HK-2) cells; AR, Androgen receptor.

of autophagy that may be conducive to the pathogenesis of polymicrobial sepsis-mediated AKI. Since then, several following preclinical studies also demonstrated a trend of rising first and then falling of autophagy during SI-AKI.

Mei et al. (47) showed that autophagy increased at 4-24h after sepsis and declined to the control level subsequently. LPS-induced renal autophagy was suppressed in Atg7-knockout animals. Additionally, more severe AKI was observed in proximal tubule-specific Atg7-knockout mice. Since the aberrant expression of autophagy-related genes significantly affects the autophagy status under sepsis, this fact may imply that autophagy plays an essential role in SI-AKI. Increased expression levels of LC3-II and reduced P62 expression at an early stage were observed in Li et al.'s study (17). The authors further pointed out that the degradation of P62 by activated autophagy at the early stages of endotoxemia might induce the inhibition of apoptosis. At the late stages of endotoxemia, inhibition of autophagy caused P62 accumulation and accelerated renal injury. Similarly, Dai et al. (49) also found that the autophagy level was first activated and then inhibited during SI-AKI. Mitophagy was increased within the first 4h after LPS stimulation and was decreased thereafter. Mitophagy protected LPS-included cells from apoptosis and improved renal functions of SI-AKI.

Under a similar trend of the autophagy change (increased first and then decreased) in SI-AKI, elevated levels of LC3, COX IV, Pink1, Parkin, and NLRP3 were identified in septic AKI (41). Impaired mitophagy in the later stage of septic AKI might be correlated with the activation of NLRP3-caspases-mediated

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TABLE 3 The characteristics and the main findings of the relevant 8 studies reporting autophagy is activated firstly and then declined in SI-AKI.

Study/Reference	Experimental model/participant	Methods for establishing sepsis-induced AKI	Status of autophagy	Associated genes or pathways	Main findings
Hsiao et al. (46)	Rat and NRK-52E	CLP	Increased autophagy in early sepsis and inhibited at 9h and 18h after CLP	Up-regulating LC3-II and Atg7	Increased LC3-II at 3h and 6 h after CLP and sequentially decreased to the basal level at 9h and 18 h after CLP. *In vitro* study, siRNA knockdown of Atg7 on NRK-52E cells significantly declined the level of LC3-II. Declination of autophagy contributed to proximal tubular dysfunction at the late stage of sepsis.
Karagiannidis et al. (39)	Rat	CLP	Autophagy increased at 6 h after sepsis and declined at 12 and 24h, while elevated at 36 h	LC3a/b and pERK expression enhanced at the early sepsis, then declined, and increased later, while pAKT expression had a contrary tendency.	Autophagy inductions might be a cytoprotective mechanism triggered under sepsis conditions, rather than an alternative cell death pathway. These results provided a new prospective in sepsis treatment.
Mei et al. (47)	Mice	LPS	Autophagy increased at 4-24h after sepsis and declined to the control level subsequently.	LC3 II was increased at 4-24h after LPS treatment, then reduced to the normal level.	LPS-induced renal autophagy is suppressed in Atg7-knockout animal. Besides, more severe AKI was observed in proximal tubule-specific Atg7-knockout mice.
Li et al. (17)	Mice	LPS	Activated firstly and then declined	Increased expression levels of LC3-II, reduced P62 expression at early stage	The degradation of P62 by activated autophagy at the early stages of endotoxemia resulted in the inhibition of apoptosis; At the late stages of endotoxemia, inhibition of autophagy caused P62 accumulation and accelerated renal injury.
Dai et al. (49)	HK-2 cells and Rat	LPS	First activated and then inhibited	Activated LC3-II, BECN-1, and PINK1-Parkin pathway	Mitophagy increased with the first 4h after LPS stimulation and was decreased thereafter. Mitophagy protected LPS-included cells from apoptosis, and improved renal functions of rats with septic AKI.
Liu et al. (25)	Mice	CLP	Elevated in early stage but declined in the later phase	Elevated levels of LC3, COX IV, Pink1, Parkin, and NLRP3	Impaired mitophagy in the later stage of septic AKI might be correlated with the activation of NLRP3-caspases-mediated proteolytic cleavage of Parkin.
Deng et al. (51)	Mice	CLP	First activated and then declined	Inhibited Beclin-1 expression	SIRT1 activation improved sepsis AKI by promoting Beclin1-mediated autophagy.
Sun et al. (52)	Mice	CLP	Activated and then returned to normal level	LC3II elevated gradually and peaked at 8 h and returned to baseline by 24 h	Sirt1 upregulation reduced sepsis-induced AKI by deacetylating p53 to activate autophagy.

AKI, Acute kidney injury; Atg7, autophagy-related gene 7; LC3, microtubule-associated protein 1 light chain 3; CLP, Cecal ligation and puncture; LPS, Lipopolysaccharide; mTOR, mechanistic target of rapamycin; Pink1, PTEN induced putative kinase 1; Parkin, Parkin RBR E3 ubiquitin protein ligase; NLRP3, NLR family pyrin domain containing 3; HK-2, Human kidney proximal tubular epithelial (HK-2) cells; Sirt1, Sirtuin 1; COX IV, cytochrome coxidase IV.

proteolytic cleavage of Parkin. Sirtuin 1 (Sirt1), an NAD^+-dependent protein deacetylase, functions to modify deacetylate histone and nonhistone proteins. Consistently, Deng et al. (51) also demonstrated that kidney autophagy was elevated in the early stage but declined in the later phase. The researchers also found that SIRT1 activation improved SI-AKI by promoting Beclin1-mediated autophagy. A recent study conducted by Sun et al. (52) indicated that the expression of LC3II elevated gradually and peaked at 8 h and returned to baseline by 24 h after SI-AKI, indicating the autophagy level increased first and then declined to normal level subsequently. The authors then showed that Sirt1 upregulation reduced SI-AKI by deacetylating p53 to activate autophagy. Differ from that of the above included studies reporting the autophagy level increased at the early stage and decreased later during SI-AKI, Karagiannidis et al. implied that the autophagy flux increased at 6 h after sepsis and declined at 12 and 24h, while elevated at 36 h. They concluded that autophagy inductions might be a cytoprotective mechanism triggered under sepsis conditions, rather than an alternative cell death pathway.

Based on the above evidence derived from *in vitro* and *in vivo* models of SI-AKI, it showed the trend of rising firstly and then falling for the autophagy flux. This may be a molecular mechanism of the renoprotective effect during SI-AKI (Figure 1, middle column). The autophagy feedback may play a protective role in endotoxic AKI, serving as a potential therapeutic target for protecting against the damage of renal tubular epithelial cells.

Hypotheses for the different level of autophagy among the included studies

Based on the different level of autophagy among the 41 included studies and outcomes of the changed autophagic flux, we propose the following hypotheses. First, the autophagic flux during the physiological processes of SI-AKI might elevate firstly (phrase I), next inhibit or decline (phrase II), then elevate (phrase III), and return to the normal level finally (phrase IV). In the 24 studies reporting the elevation of autophagy, it could be explained by the check point time of autophagy in these studies was phrase I or phrase III. The eight studies reporting the elevation first then inhibition could be explained by check point time of autophagy was phrase I and phrase II of SI-AKI. Of note, in the nine included studies reported elevated first and then declined, all of them concluded that the autophagy status was inhibited during SI-AKI, while the renal protective effects exerting by specific interventions (reported in eight studies) might be contributed to the elevation of autophagy. Therefore, the check point time of autophagy in these included studies might be the phrase II. The above interventions exerted the renoprotective effects on SI-AKI might be associated with the acceleration of autophagy from phrase II to phrase III.

The changes of autophagic flux under different interventions in the experimental models of SI-AKI

As shown in the Table 4, the majority of the included studies (14/15, 93%) showed that the improvement of SI-AKI exhibited by specific interventions might be attributed to the elevation of autophagy, regardless of whether the autophagy activated or inhibited during SI-AKI. The most likely explanation is the elevation of autophagy may be one of the protective mechanisms for SI-AKI. Another possible explanation is that the activation and inhibition of autophagy among different studies might be associated with the different time points of examinations after sepsis in each independent study. As illustrated in Table 3, eight included studies indicated the autophagy was activated firstly (early stage of SI-AKI) and then declined (late stage of SI-AKI) during SI-AKI. Therefore,

TABLE 4 The status of autophagy under different interventions in the experimental models of SI-AKI.

Study/ Reference	Experimental model/ participant	Methods for establishing sepsis-induced AKI	Specific interventions	Status of autophagy without interventions	Status of autophagy under interventions
Jia et al. (19),	Rat	CLP	Alpha-Lipoic Acid	Activated	Activated
Gao et al. (24),	Mice	CLP	Polydatin	Activated	Activated
Chen et al. (28),	Mice and HK-2 cells	LPS	Ascorbate	Activated	Activated
Guo et al. (29)	Rat, HK-2, RTECs cells	CLP and LPS	BMSCs	Activated	Activated
Tan et al. (33)	Mice	CLP	Aerobic glycolysis	Activated	Activated
Li et al. (35)	Mice and HK-2 cells	LPS	Sodium hydrosulfide hydrate	Activated	Activated
Li et al. (37)	Rat	CLP	Ulinastatin	Activated	Inhibited
Howell et al. (38)	Mice	CLP and LPS	temsirolimus	Inhibited	Activated
Sunahara et al. (48)	Mice	CLP	Rapamycin	Inhibited	Activated
Li et al. (40)	HK-2 and HEK-293 cells	LPS	Recombinant human erythropoietin	Inhibited	Activated
Feng et al. (23)	Rat	LPS	umbilical cord blood mononuclear cells	Inhibited	Activated
Liu et al. (25)	Mice	LPS	Procyanidin B2	Inhibited	Activated
Yang et al. (43)	Rat	LPS	Dexmedetomidine	Inhibited	Activated
Zhao et al. (44),	Rat	LPS	Dexmedetomidine	Inhibited	Activated
Yu et al. (45)	Mice	LPS	NF-κB inhibitor 270	Inhibited	Activated

those studies listed in Table 1 suggested that autophagy was activated, which might be due to the time point for checking the autophagy level being the early stage. On the other hand, the inhibition of autophagy in the studies listed in Table 2 might be correlated to the checking time point was the late stage of SI-AKI.

In Table 1, there were seven studies reported some interventions for treating SI-AKI. All of the seven included studies indicated CLP or LPS treatment (methods for SI-AKI model establishment) might induce the elevation of autophagy level. Six of them reported that the specific interventions (i.e., alpha-lipoic acid, Polydatin, Ascorbate, BMSCs, aerobic glycolysis, and sodium hydrosulfide hydrate) exhibited the renal protection by increasing autophagy. In Table 2, eight studies reported some interventions for treating SI-AKI. Inconsistent with the above findings, all the eight studies concluded that the autophagy status was inhibited during SI-AKI, while the renal protective effects exerting by specific interventions (i.e., temsirolimus, rapamycin, recombinant human erythropoietin, umbilical cord blood mononuclear cells, Procyanidin B2, dexmedetomidine, and NF-κB inhibitor 270) might be contributed by the elevation of autophagy.

Based on the above evidence, in the aspect of the clinical translational perspective, the strategies for therapeutic intervention should focus on how to elevate the autophagic flux during SI-AKI.

Conclusion and perspectives

To the best of our knowledge, this is the first systematic and comprehensive review to summarize all the current evidence of the crucial roles of autophagy in SI-AKI. We can notice that a majority of the included studies (about 60%) showed an elevation of the autophagy level during SI-AKI, while 22% and 19.5% of the included studies reported an inhibition and an elevation at the early stage but a declination of renal autophagy in SI-AKI, respectively. As can be seen, the level of autophagy flux in the process of septic AKI is still controversial among different studies. One of the explanations for this inconsistency of the autophagy level in SI-AKI may be caused by the various time points monitored in each study. In addition, different intracellular signaling molecules and pathways involved in the process of SI-AKI may also affect the expression of the autophagy-related genes, resulting in an increase or decrease of autophagy flux. Autophagy is considered to be a "double-edged sword" for both cell survival and cell death in multiple diseases, including SI-AKI. However, this study highlights that one of the main probable mechanisms underlying the multiple treatment modalities (e.g. small molecule inhibitors, temsirolimus, rapamycin, ascorbate, rhEPO, stem cells, Procyanidin B2, and DEX) for improving the renal function may be attributed to the elevation of the autophagy level in SI-AKI. The exact roles of autophagy in SI-AKI have not been well understood, which deserves further investigation. Targeting the autophagy-associated proteins and pathways may provide a new prospective in the treatment of critically ill patients with SI-AKI, but more preclinical studies are still warranted to validate this hypothesis.

Author contributions

SZ, JL, and MW contributed to conceiving and designing the study. XL performed the systematic searching. MLS extracted the data. SZ and JL wrote the manuscript. XL and MW supervised the manuscript. All authors contributed to the article and approved the submitted version.

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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.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2023.1180866/full#supplementary-material

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Glossary

Atg7	autophagy-related gene 7
LC3	microtubule-associated protein 1 light chain 3
CLP	Cecal ligation and puncture
LPS	Lipopolysaccharide
mTOR	mechanistic target of rapamycin
Pink1	PTEN induced putative kinase 1
Parkin	Parkin RBR E3 ubiquitin protein ligase
NLRP3	NLR family pyrin domain containing 3
HK-2	Human kidney proximal tubular epithelial (HK-2) cells
BMSCs	Bone marrow-derived mesenchymal stem cells
RTECs	Renal tubular epithelial cells
RIP3	Receptor interacting protein kinase 3
ATM	ataxia-telangiectasia mutated
15-PGDH	15-hydroxyprostaglandin dehydrogenase
TREM-1	Triggering receptor expressed by myeloid cells
TLR4	Toll Like Receptor 4
Pink1	PTEN induced putative kinase 1
Parkin	Parkin RBR E3 ubiquitin protein ligase
NLRP3	NLR family pyrin domain containing 3
HK-2	Human kidney proximal tubular epithelial (HK-2) cells
AR	Androgen receptor
COX IV	cytochrome coxidase IV

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