Elevated MIF identified by multiple cytokine analyses facilitates macrophage M2 polarization contributing to postoperative recurrence in chronic rhinosinusitis with nasal polyps

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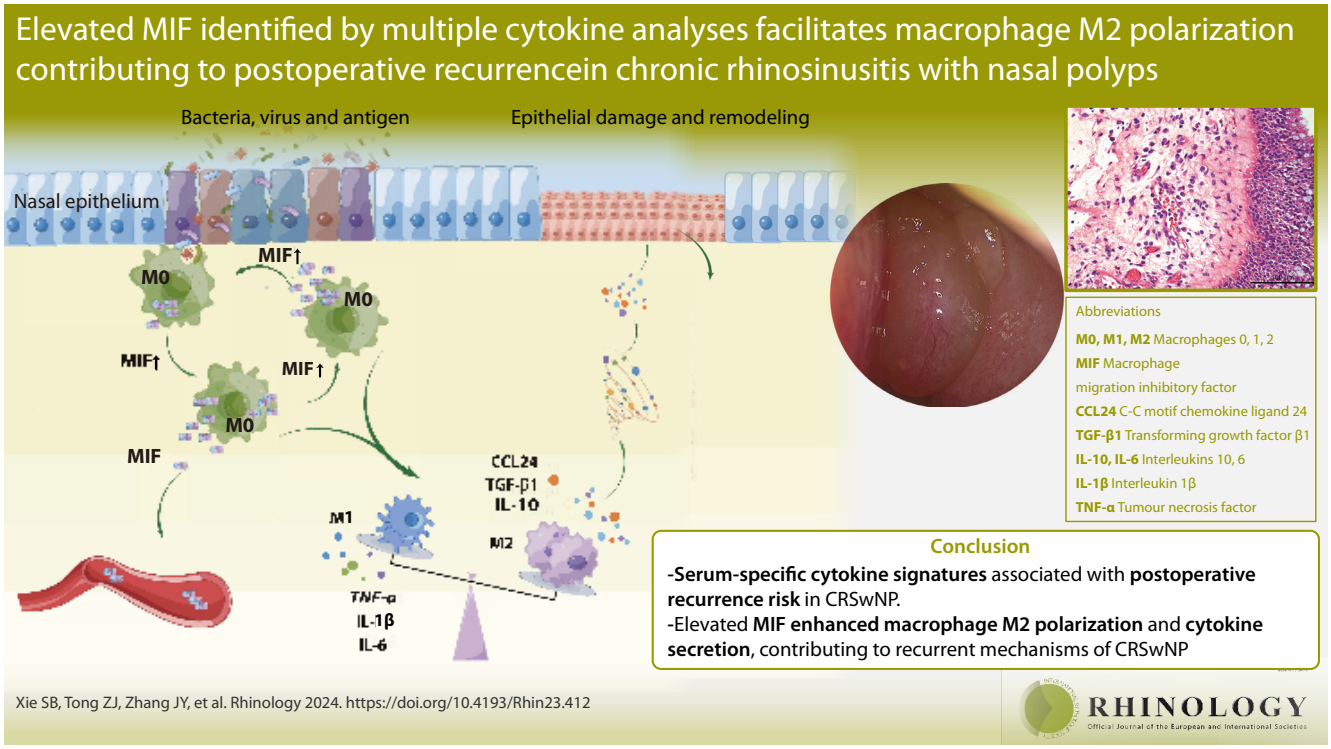

Abstract

Background: Chronic rhinosinusitis with nasal polyps (CRSwNP) is characterized by tissue heterogeneity and high postoperative recurrence risk. This study aims to employ cytokine analyses to identify serum biomarkers associated with postoperative CRSwNP recurrence and elucidate underlying recurrent mechanisms. **Methods:** A prospective cohort study was conducted on CRSwNP patients undergoing functional endoscopic sinus surgery. Serum and tissue samples were collected and analyzed for multiple cytokines. Participants were followed for 3 years and categorized into recurrent and non-recurrent groups. Cytokine profiles were compared, and potential markers for recurrence were further assessed. Macrophage migration inhibitory factor (MIF) expression in macrophages was modulated, and their polarization and cytokine secretion were assessed. **Results:** In the discovery cohort (21 recurrent and 40 non-recurrent patients), circulating cytokine profiles differed significantly, with 8 cytokines showing differential expression between the two groups. Among them, serum eotaxin, MIF, RANTES, and TRAIL exhibited promise in predicting recurrence. In the validation cohort (24 recurrent and 44 non-recurrent patients), serum eotaxin, MIF, and TRAIL levels were higher in recurrent cases. Tissue MIF was elevated in recurrent cases and had a strong predictive value for recurrence. Moreover, tissue MIF was co-expressed with CD206 in recurrent cases. Mechanistically, MIF overexpression promoted macrophage M2 polarization and TGF-β1, CCL-24, and MIF secretion, and MIF recombinant protein facilitated M2 polarization, and TGF-β1 and CCL-24 production, contributing to CRSwNP recurrence. **Conclusion:** Serum-specific cytokine signatures were associated with postoperative recurrence risk in CRSwNP. Elevated MIF enhanced macrophage M2 polarization and cytokine secretion, contributing to the recurrent mechanisms of CRSwNP.

Key words: chronic rhinosinusitis with nasal polyps, macrophage migration inhibitory factor, macrophage, cytokine, recurrence

Introduction

Chronic rhinosinusitis with nasal polyps (CRSwNP) is a debilitating and recurrent inflammatory condition of the nasal and paranasal sinus mucosa [1]. Currently, the management of CRSwNP primarily encompasses medical interventions, biological therapies, and functional endoscopic sinus surgery (FESS) [2, 3]. Patients who exhibit inadequate responses to conservative treatments often choose FESS as an alternative [4, 5]. Although FESS has demonstrated the potential to improve the quality of life for individuals with CRSwNP, a proportion of patients continue to encounter postoperative recurrence because of the complex etiology and pathogenic mechanisms [6, 7]. Therefore, exploring early predictive biomarkers for postoperative recurrence in CRSwNP and elucidating their potential mechanisms of recurrence can aid in developing targeted treatment strategies, ultimately achieving individualized precision therapy.

The pathophysiology of CRSwNP is characterized by intricate interactions among a variety of immune and inflammatory cells, creating a complex immunological microenvironment that influences the development and reappearance of nasal polyps [8, 9]. Central to these processes are cytokines, which act as signaling molecules orchestrating immune responses and inflammation [10, 11]. Consequently, analyzing cytokine levels in the bloodstream provides valuable insights into the systemic immune profile of CRSwNP patients and their predisposition to postoperative recurrence. Previous research has found an association between abnormal cytokine concentrations in both tissue and peripheral blood and the prognosis and postoperative recurrence of CRSwNP, including IL-13 [12], IL-5 [13], and periostin [14]. However, there has been relatively little systematic investigation into the relationship between the expression profile of multiple circulating cytokines and the risk of postoperative recurrence in CRSwNP.

To fill this knowledge gap, we conducted a prospective cohort study involving the analysis of 48 cytokines to investigate potential serum cytokines as predictive biomarkers for CRSwNP recurrence. We identified the most promising cytokines through screening and subsequently validated their significance using serum and tissue samples from an independent validation cohort. Furthermore, we elucidated their underlying mechanisms through in vitro testing.

Materials and methods

Patients and settings

This prospective study consists of two independent cohorts, a discovery, and a validation cohort. The discovery cohort initially enrolled 72 CRSwNP patients and the validation cohort initially recruited 80 CRSwNP patients who underwent FESS between May 2019 and August 2019. CRSwNP was diagnosed according to the European Position Paper on Rhinosinusitis and Nasal Polyps 2012 [15]. Patients were excluded from the study if they had incomplete clinical data; were diagnosed with fungal rhinosinusitis, allergic fungal rhinosinusitis, or sinonasal tumors; had received oral corticosteroids, antibiotics, or other immune-regulating drugs within the last month; had acute inflammation or asthma; or were either younger than 18 years or older than 65 years of age. The research protocol received approval from the Human Ethical Committee at Xiangya Hospital of Central South University (No.201907812). Before their involvement in the study, all patients furnished written informed consent.

Patient follow-up

All CRSwNP underwent FESS conducted by three experienced surgeons, adhering to consistent surgical standards. After surgery, patients were prescribed fluticasone propionate nasal spray and saline irrigation twice daily as part of their routine care. For those symptoms that remained uncontrolled, a course of broad-spectrum antibiotics (oral cephalosporin) was administered for 1-2 weeks, and oral prednisone tablets for 3 weeks were available as an option as previously described [12, 16]. Regular follow-up appointments were scheduled to monitor disease progression through endoscopic examinations and debridement. A minimum follow-up period of 3 years was maintained for all patients, who were categorized into recurrent and non-recurrent groups. The criteria for postoperative recurrence included the persistence of reappearance of clinical symptoms, endoscopic signs, and/or computed tomography (CT) findings for a minimum of 2 months, despite the implementation of the previously described antibiotic and oral steroid rescue regimen [7, 12]. Upon confirmation of postoperative recurrence, the follow-up schedule is terminated, and the current time is designated as the concluding follow-up time.

Serum sample collection and multiple cytokine detection

Serum samples were collected from CRSwNP patients before FESS and immediately stored at -80°C for subsequent experiments. Serum cytokine levels were measured using a commercial multiplex assay kit (BioRad, CA, USA), and analyzed in the Luminex system. The Plex Panel kit included 48 cytokines, and their full names, abbreviations, and detection limits are listed in Table S1. All serum samples were subjected to a twofold dilution to ensure that the detection limits of cytokine assessments were fully covered. The assays were conducted following the manufacturer's protocol, enabling the determination of cytokine levels with coefficients of variation of less than 15%, as previously outlined in our study [17]. The results obtained from the Bio-Plex assays were imported into an Excel spreadsheet for data analysis. When interpreting the data, cytokine values that fell below the detection limit were estimated using robust regression on order statistics, as explained in our previous descriptions [17].

Table 1. Baseline characteristics of CRSwNP patients in the discovery cohort.

	Recurrent group	non-Recurrent group	P value
Number, (n)	21	40	
Male/female	14/7	26/14	0.896
Age, months	35.0 (27.0, 50.5)	44.5 (27.3, 46.8)	0.230
BMI, kg/m^2	23.6±2.7	23.1±2.2	0.863
Smoking, n (%)	9 (42.9)	11 (27.5)	0.225
Alcohol consumption, n (%)	3 (14.3)	8 (20.0)	0.581
Duration of disease, months	36.0 (12.0, 72.0)	48.0 (27.0, 72.0)	0.952
Allergic rhinitis, n (%)	6 (28.6)	8 (20.0)	0.449
Asthma, n (%)	2 (9.5)	4 (10.0)	1.000
VAS	8.0 (7.5, 9.5)	8.0 (7.0, 10.0)	0.983
Lund-MacKay score	13.0 (11.0, 15.0)	12.0 (11.0, 14.0)	0.656
Lund-Kennedy score	7.0 (6.5, 9.0)	7.0 (6.0, 8.0)	0.339
Follow-up, months	18.0 (12.0, 24.0)	42.0 (36.0, 45.0)	<0.001

CRSwNP, chronic rhinosinusitis with nasal polyps; BMI, body mass index; VAS, visual analogue score.

Enzyme-linked immunosorbent assay (ELISA)

Cytokines identified as potential candidates through multiple cytokine analyses were subsequently validated in the validation cohort using enzyme-linked immunosorbent assay (ELISA). ELISA was also employed to determine the concentrations of cytokines in the cell supernatants. Eotaxin, macrophage migration inhibitory factor (MIF), regulated on activation in normal T-cell expressed and secreted (RANTES), and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), tumor necrosis factor (TNF) -α, interleukin (IL)-1β, IL-12, transforming growth factor (TGF)-β1, chemokine (C-C motif) ligand (CCL)-24, and IL-10 ELISA kits were purchased from Multisciences (Hangzhou, China) in adherence to the manufacturer's instructions, with the operators of the assay remaining unaware of the specific subject data. Duplicate measurements were taken for all samples, and the mean values were employed in the analysis.

Real-time quantitative PCR analysis (RT-qPCR)

Tissue samples were collected during FESS and stored in liquid nitrogen. The total RNA of tissue samples was extracted with Trizol (Thermo Fisher Scientific, USA), and the concentrations were detected before reverse transcription. Subsequently, we applied the 20 μL system to perform reverse transcription to generate cDNA. The mRNA expression of the candidate genes was detected using the TaqMan® Gene Expression Kit (Thermo Fisher Scientific), with GAPDH as the reference gene. The PCR program adhered to the guidelines provided by the reagent instructions. The RT-qPCR results were represented as the fold change in gene expression, which was normalized to the reference gene GAPDH. Basal expression values were determined using the Ct method (ΔΔCT method). The primer sequences can be found in Table S2.

Immunohistochemistry (IHC) staining

The nasal polyp tissues were harvested during FESS and fixed in 4 % paraformaldehyde for 48 h and embedded in a wax block. The tissue blocks were sectional into 5 μm sections, and the paraffin sections were dewaxed, rehydrated, and treated with antigen retrieval buffer as previously described [18]. The sections were incubated with H_2O_2 for 10 min, then blocked with 10% goat serum for 30 min at room temperature. The blocked sections were incubated with anti-eotaxin (1:200, Affinity, China), anti-MIF (1:200, Affinity), anti-RANTES (1:200, Affinity), and anti-TRAIL (1:200, Affinity) overnight at 4°C. The next day, the sections were washed extensively with PBS, and incubated with HRP-conjugated secondary antibody (1:200, Affinity) for 1 h followed by colorimetric detection with a DAB kit (Sigma, USA) and counterstained with hematoxylin, followed by dehydration through a graded ethanol series, and xylene transparent. For the data quantification, the integrated optical density (IOD) of positive expression in tissues was measured by ImageJ software, and the values of IOD/area were applied for comparison. All negative controls were incubated with phosphate-buffered saline (PBS) instead of the primary antibody, and the images were listed in Figure S1.

Multiple immunofluorescence

The multiple fluorescence staining was performed using the Dual-label Multiplex Immunoassay Kit (Aifang, China) following the provided protocol. Briefly, paraffin-embedded sections were sectioned, followed by dewaxing, rehydration, and antigen retrieval as described before. Subsequently, the sections were

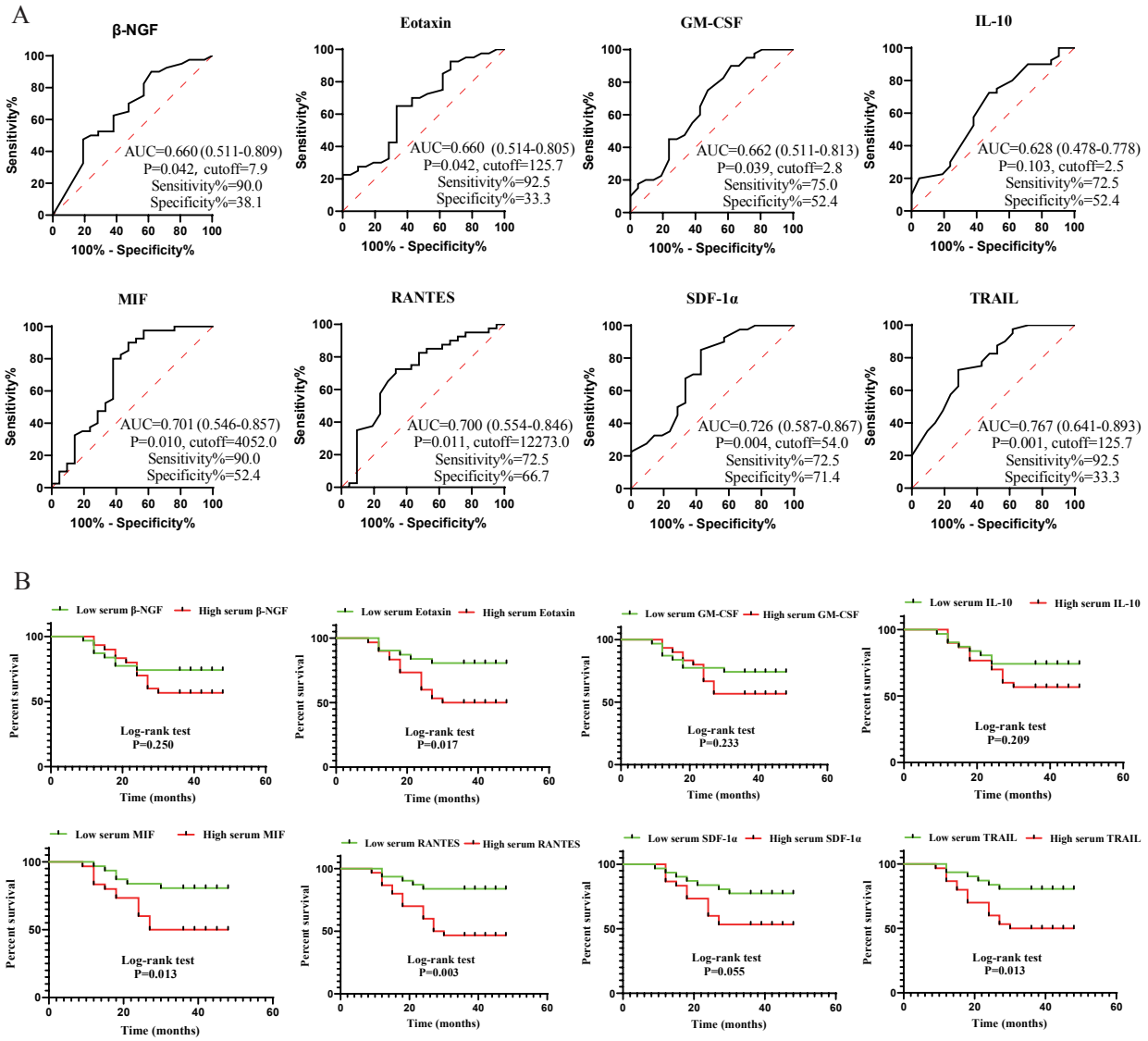

Figure 1. The predictive values of 8 candidate serum cytokines for CRSwNP recurrence in the discovery cohort. (A) ROC analysis; (B) Kaplan-Meier survival. High and low serum cytokine levels were defined on their median values. CRSwNP, chronic rhinosinusitis with nasal polyps; ROC, receiver operating characteristic.

immersed in H_2O_2 for 10 min and then subjected to blocking with 10% goat serum for 30 min. Tissue sections were incubated with the primary anti-MIF antibody (1:200, Affinity) overnight at 4°C, followed by treatment with an HRP-labeled secondary antibody (1:200, Affinity). After thorough washing, antigen retrieval was conducted again, and the sections were then incubated with primary antibodies against NOS2 or CD206 (1:200, Affinity) overnight at 4°C. Subsequently, the sections were exposed to Cy3-labeled fluorescent secondary antibodies (Abcam, UK) for 1 h. DAPI solution was applied to each section to visualize the cell nuclei. All slides were subsequently covered with glass coverslips and examined under a fluorescence microscope. The quantification of co-expressed positive cells was performed in high-power fields. All negative controls were incubated with PBS instead of primary antibodies, and the images were presented in Figure S2.

Culture and intervention of peripheral blood macrophages

The human macrophages were induced from peripheral blood mononuclear cells (PBMC) isolated from healthy control (HC) as previously described [19]. Subsequently, the PBMCs were incubated with anti-CD14 magnetic beads to sort monocytes, and the collected monocytes were cultured in RPMI-1640 medium (Gibco, USA) supplemented with 10% fetal bovine serum (Gibco). To induce differentiation into macrophages, the monocytes were treated with 100 ng/ml of M-CSF (Prospect, UK) at 37°C with 5% CO_2 for 5 days, as previously described [19]. The resulting macrophages derived from PBMCs were harvested and further maintained in culture. To overexpress MIF in macrophages, the macrophages were starved in an FBS-free medium for

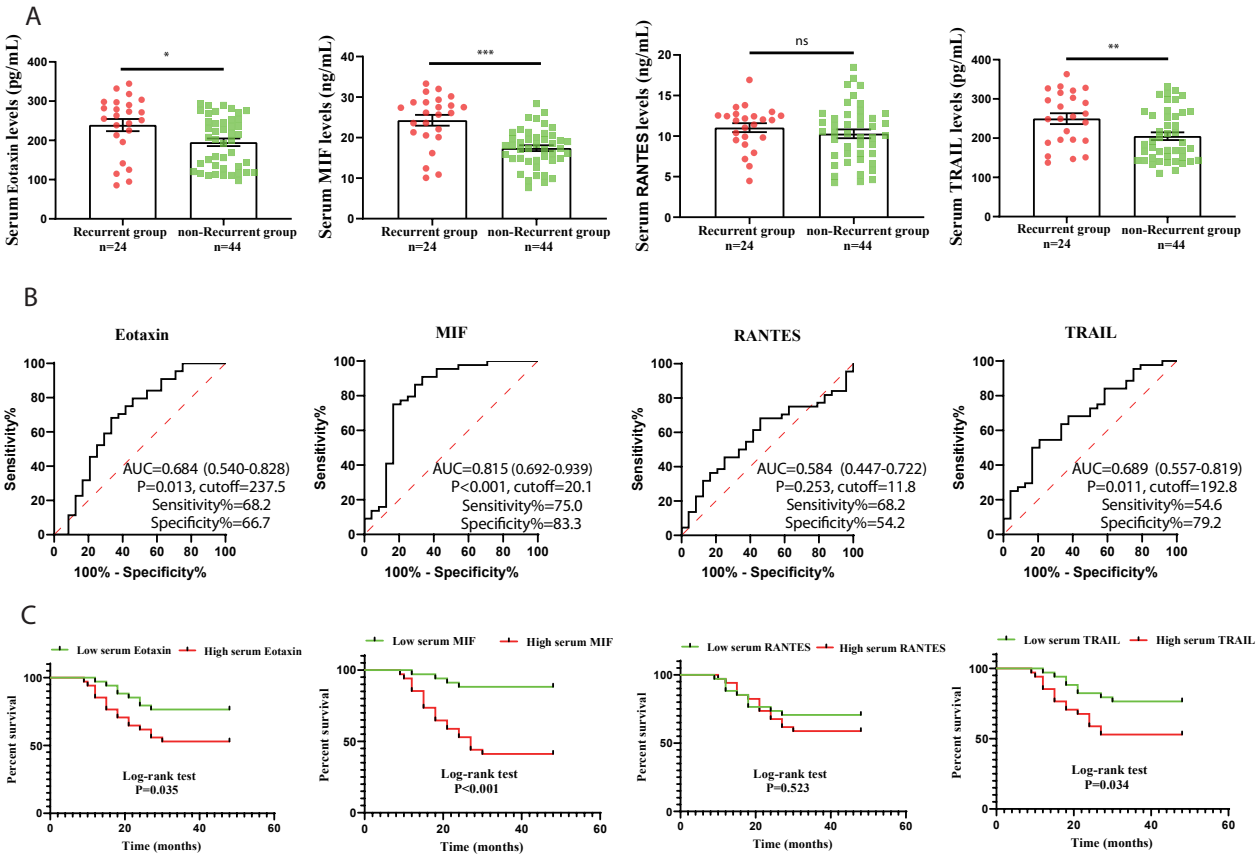

Figure 2. The abilities of 4 potential serum cytokines for predicting CRSwNP recurrence in the validation cohort. (A) the comparisons of serum eotaxin, MIF, RANTES, and TRAIL concentrations between recurrent and non-recurrent CRSwNP patients. (B) ROC analysis; (C) Kaplan-Meier survival analysis. High and low serum cytokine levels were defined on their median values. CRSwNP, chronic rhinosinusitis with nasal polyps; ROC, receiver operating characteristic. *P<0.05, **P<0.01, ***P<0.001, ns, no significance.

24 h, then transfected with MIF over-expression (OE) plasmid (Genechem, China) using the reagent Lipofectamine 3000 (Invitrogen, USA) for 6 h. To investigate the impact of exogenous MIF on macrophages, we stimulated macrophages with 50 ng/ml recombinant MIF protein (Solarbio, China) for 24 h as previously described [20, 21]. All treated cells and cell supernatants were collected for subsequent experiments.

Western blotting (WB)

Cell total proteins were extracted using RIPA lysis buffer (Beyotime, China), followed by protein concentration detection with the BCA protein assay kit (Beyotime). Proteins were treated with loading buffer and electrophoresed on SDS polyacrylamide gels and then transferred onto polyvinylidene fluoride (PVDF) membranes (Thermo Fisher). After incubation with 5% skim milk for 1 h, the membranes were incubated overnight at 4°C with the corresponding primary antibody against CD86, NOS2, CD163, CD206, and Tubulin (Affinity) in a dilution ratio of 1:1000. After being washed with TBST for 3 times, the membranes are incubated with the secondary antibody (Affinity) in a dilution ratio of 1:3000 for 1 h at room temperature. Protein bands were adequately washed with TBST and then detected by ECL ultrasensitive luminescent phenol (Beyotime). Their intensities of bands were measured by Image J software, and the target proteins were normalized to Tubulin to determine their relative expression levels.

Statistical analysis

Continuous variables with normal distribution are expressed as mean ± SD and analyzed with Student's t-test. Continuous variables with normal distribution were reported as median and interquartile ranges (IQRs), and the Mann-Whitney U test was employed for comparison. Categorical data were presented in terms of frequencies and percentages, and statistical differences were assessed using the chi-square test. Spearman test was performed for correlation analysis. For experimental data in GraphPad Prism, the mean ± standard error of the mean (SEM) was reported, and the Mann-Whitney U test was utilized for analysis. Receiver operating characteristic curve (ROC) analyses were conducted to evaluate the predictive potential of cytokines in CRSwNP recurrence. Patients were categorized into high and low-level groups based on the median values of cytokine levels.

The median values served as the cutoff points, with expression levels exceeding the cutoff being included in the high-level group, while those below were assigned to the low-level group. The association between cytokine levels and the risk of CRSwNP recurrence was assessed using Kaplan-Meier survival analysis. Data analysis was performed using SPSS 23.0 (SPSS, USA) and GraphPad Prism 8.0 (GraphPad, USA). A significance level of 0.05 was applied for all tests, with P values below this threshold considered statistically significant.

Results

Clinical characteristics of all subjects

In the discovery cohort, 61 CRSwNP patients completed the 3-year follow-up schedule, while 11 patients withdrew from the study. Among these recruited patients, 21 patients were included in the recurrent group and 40 patients were classified into the non-recurrent group. There were no significant differences found between the two groups in gender, age, BMI, alcohol consumption, disease duration, history of allergic rhinitis or asthma, and the Lund-MacKay and Lund-Kennedy scores, except for variations in follow-up time (Table 1).

Distinctive serum cytokine profiles associated with postoperative recurrence in CRSwNP

The serum 48-cytokine analysis results revealed distinctive cytokine profiles between the recurrent and non-recurrent groups. The results found in Table 2 illustrated that 8 out of 48 tested cytokines were abnormally expressed in the recurrent group alone, and not in the non-recurrent group. The serum concentrations of β-NGF, eotaxin, GM-CSF, IL-10, MIF, RANTES, SDF-1α, and TRAIL were increased in the recurrent group in comparison with the non-recurrent group. The ROC curves showed that β-NGF, eotaxin, GM-CSF, MIF, RANTES, SDF-1α, and TRAIL exhibited varying degrees of predictive capabilities for CRSwNP recurrence (Figure 1A). Kaplan-Meier survival analysis suggested that elevated serum eotaxin, MIF, RANTES, and TRAIL levels were associated with the risk of postoperative recurrence in CRSwNP patients (Figure 1B).

Increased MIF aggravates the risk of postoperative recurrence in CRSwNP

The potential four cytokines (eotaxin, MIF, RANTES, and TRAIL) were further validated in the validation cohort. The validation cohort finally comprises 44 non-recurrent CRSwNP patients and 24 recurrent CRSwNP patients after 3 years of follow-up, and the baseline characteristics of subjects are shown in Table 3. The ELISA results in Figure 2A revealed that serum eotaxin, MIF, and TRAIL levels were increased in the recurrent group compared to the non-recurrent group. The ROC and Kaplan-Meier survival analysis suggested that serum eotaxin, MIF, and TRAIL levels were positively associated with the risk of postoperative recurrence (Figure 2B-C).

To further confirm the associations between cytokines and postoperative recurrence of CRSwNP, we evaluated the tissue expressions of these four cytokines. The RT-qPCR results in Figure 3A showed that tissue eotaxin, MIF, and RANTES expressions were enhanced in the recurrent group compared to the non-recurrent group. Moreover, the correlation analysis demonstrated that only tissue MIF expression was positively correlated with serum MIF concentrations in CRSwNP patients (Figure S3). The IHC staining results showed that tissue eotaxin and MIF were significantly enhanced in the recurrent group in comparison with the non-recurrent group (Figure 3B). Additionally, our findings indicated that CRSwNP patients in the revised surgery group displayed higher tissue MIF expression when compared to those in the primary surgery group. This suggests that recurrent tissues showed higher MIF levels compared to their baseline levels, and elevated MIF may contribute to the recurrent mechanisms of CRSwNP.

MIF promotes macrophage M2 polarization contributing to CRSwNP recurrence

Prior studies have shown that MIF can influence macrophage function and that the polarization of macrophages plays an essential role in the development of CRSwNP [22-24]. We assessed the tissue expressions of macrophage polarization markers and observed that CD86, CD163, and CD206 were elevated in the recurrent group in comparison to the non-recurrent group. Furthermore, the tissue MIF levels were shown to have a positive association with the expressions of CD163 and CD206, indicating a correlation between MIF and macrophage M2 polarization (Figure S4). The multiplex immunofluorescence analysis in Figure 4 revealed a co-expression of MIF with CD206, and the count of MIF^+CD206^+ cells was notably higher in the recurrent group when compared to the non-recurrent group. Additionally, tissues obtained during the revised surgery showed a greater presence of MIF^+CD206^+ cells than those collected during the primary surgery from the same patients, as depicted in Figure 5. These findings indicate that M2 polarization plays a predominant role in the histopathology of recurrent CRSwNP, and MIF may promote M2 polarization, thereby contributing to the mechanism of recurrence.

To delve deeper into the impact of MIF on M2 polarization, we augmented MIF expression in macrophages using plasmids. The WB results demonstrated that CD163 and CD206 were enhanced after up-regulation of MIF, while no significant influence was observed in CD86 and NOS2 (Figure 6A-B). ELISA results demonstrated a significant increase in the concentrations of TNF-α, TGF-β1, CCL-24, IL-10, and soluble MIF in cell supernatant in the OE group in comparison with the NC group (Figure 6C). Moreover, we employed MIF recombinant protein to stimulate macrophages, leading to an increase in CD163 and CD206

Table 2. Comparison of serum 48 cytokines levels between the two groups (pg/ml).

Cytokines	Recurrent group (n=21)	non-Recurrent group (n=40)	P value
Basic FGF	48.5 (45.1, 59.8)	48.5 (42.2, 59.4)	0.611
β-NGF	1.6 (0.3, 12.1)	0.2 (0, 3.9)	0.024
CTACK	1953.5 (1590.3.1, 2710.0)	1811.0 (1543.5, 2413.0)	0.458
Eotaxin	87.9 (56.4, 135.7)	70.8 (57.2, 99.0)	0.046
G-CSF	281.0 (164.1, 357.0)	170.8 (102.4, 296.7)	0.223
GM-CSF	3.1 (1.5, 4.6)	1.9 (1.4, 3.0)	0.006
GRO-α	731.4 (642.9, 789.1)	615.1 (540.1, 696.7)	0.162
HGF	787.6 (420.4, 907.2)	607.3 (463.3, 896.9)	0.893
IFN-α2	4.7 (1.3, 9.3)	1.4 (0.5, 4.4)	0.260
IFN-γ	10.1 (8.0, 13.2)	9.5 (8.2, 12.3)	0.258
IL-10	2.6 (1.9, 4.0)	2.0 (1.7, 3.1)	0.046
IL-12(p40)	42.5 (6.8, 86.8)	26.0 (5.2, 79.5)	0.490
IL-12(p70)	3.3 (2.5, 3.6)	2.9 (2.5, 3.5)	1.000
IL-13	2.4 (1.5, 4.7)	1.9 (1.5, 2.4)	0.126
IL-15	7.0 (0.2, 41.2)	0.8 (0.1, 26.0)	0.793
IL-16	266.4 (153.3, 443.8)	201.8 (133.2, 322.0)	0.652
IL-17	12.9 (11.5, 15.0)	11.3 (9.8, 12.9)	0.729
IL-18	97.2 (55.4, 108.3)	67.2 (44.6, 111.9)	0.926
IL-1α	22.8 (9.3, 39.7)	21.7 (13.3, 36.1)	0.532
IL-1β	4.6 (3.3, 6.5)	4.4 (3.2, 6.1)	0.811
IL-1ra	1014.0 (781.9, 1945.9)	834.4 (455.3, 1200.6)	0.262
IL-2	1.7 (0.9, 3.2)	1.4 (0.6, 2.1)	0.282
IL-2R α	164.0 (104.1, 175.0)	127.8 (99.6, 204.8)	0.251
IL-3	0.1 (0, 0.1)	0.1 (0, 0.2)	0.330
IL-4	3.7 (3.2, 4.6)	3.5 (2.8, 4.3)	0.478
IL-5	0.3 (0.2, 1.7)	0.8 (0.4, 1.6)	0.403
IL-6	0.7 (0.3, 1.4)	0.3 (0.1, 1.0)	0.404
IL-7	12.6 (6.0, 16.1)	6.8 (5.3, 11.4)	0.348
IL-8	190.1±150.4	186.0±209.8	0.937
IL-9	478.6 (448.5, 499.8)	468.3 (438.8, 489.0)	0.254
IP-10	675.4 (489.1, 934.6)	590.1 (461.1, 783.8)	0.926
LIF	94.5 (84.9, 107.8)	94.5 (78.6, 118.6)	0.501
MCP-1	115.1 (64.8, 195.6)	71.1 (49.5, 106.4)	0.479
MCP-3	0.5 (0, 13.5)	0 (0, 5.9)	0.900
M-CSF	67.8±72.6	58.3±48.5	0.542
MIF	4140.0 (1983.0, 6391.0)	2308.0 (1448.4, 2976.0)	<0.001
MIG	317.8 (260.1, 608.1)	382.0 (261.5, 516.0)	0.843
MIP-1α	18.7 (12.4, 27.7)	13.4 (7.3, 22.6)	0.313
MIP-1β	321.9 (279.7, 418.1)	295.2 (267.8, 339.8)	0.254
PDGF-BB	4036.9±1549.5	3938.3±2163.8	0.854
RANTES	12637.7±2198.5	11461.6±1722.1	0.025
SCF	156.6±45.7	145.1±42.2	0.329
SCGF-β	298642.0 (276854.0, 422740.0)	332802.0 (275355, 378992.0)	0.412
SDF-1α	2150.0 (1750.6, 2319.0)	1667.0 (1463.8, 2052.5)	0.003

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Table 2 continued.

Cytokines	Recurrent group (n=21)	non-Recurrent group (n=40)	P value
TNF-α	43.4 (31.8, 52.3)	33.0 (27.3, 43.9)	0.145
TNF-β	452.7 (419.7, 478.0)	433.6 (406.3, 462.7)	0.375
TRAIL	58.9 (50.0, 75.5)	49.5 (44.4, 56.1)	<0.001
VEGF	9.3 (0, 78.1)	0.1 (0, 11.1)	0.148

FGF, fibroblast growth factor; NGF, nerve growth factor; CTACK, cutaneous T cell attracting chemokine; G-CSF, granulocyte colony stimulating factor; GM-CSF, granulocyte-macrophage colony stimulating factor; GRO, growth-regulated oncogene; HGF, hepatocyte growth factor; IFN, interferon; IL, interleukin; IP, interferon-inducible protein; LIF, leukemia inhibitory factor; MCP, monocyte chemotactic protein; M-CSF, macrophage colony stimulating factor; MIF, macrophage migration inhibitory factor; MIG, monokine induced by interferon-gamma; MIP, macrophage inflammatory protein; PDGF, platelet-derived growth factor; RANTES, regulated on activation in normal T-cell expressed and secreted; SCF, stem cell factor; SCGF, stem cell growth factor; SDF, stromal cell-derived factor; TNF, tumor necrosis factor; TRAIL, tumor necrosis factor related apoptosis inducing ligand; VEGF, vascular endothelial cell growth factor.

Table 3. Baseline characteristics of CRSwNP patients in the validation cohort.

	Recurrent group	non-Recurrent group	P value
Number, (n)	24	44	
Male/female	14/10	27/17	1.000
Age, years	42.5 (26.0, 54.0)	42.0 (34.3, 50.8)	0.765
BMI, kg/m^2	22.8±2.3	23.7±2.4	0.172
Smoking, n (%)	8 (33.3)	11 (25.0)	0.574
Alcohol consumption, n (%)	3 (12.5)	8 (18.2)	0.734
Duration of disease, months	36.0 (12.0, 72.0)	48.0 (27.0, 72.0)	0.154
Allergic rhinitis, n (%)	8 (33.3)	10 (22.7)	0.343
Asthma, n (%)	5 (20.8)	6 (13.6)	0.441
VAS	8.0 (7.0, 9.0)	9 (7.0, 9.0)	0.598
Lund-MacKay score	13.0 (11.0, 14.0)	12.0 (11.3, 14.0)	0.723
Lund-Kennedy score	7.0 (5.3, 8.8)	8.0 (6.0, 9.0)	0.985
Follow-up, months	14.0 (12.8, 24.0)	36.0 (21.8, 42.0)	<0.001

CRSwNP, chronic rhinosinusitis with nasal polyps; BMI, body mass index; VAS, visual analogue score.

expression. Additionally, the levels of IL-1β, TGF-β1, and CCL-24 in the cell supernatant were elevated following MIF stimulation (Figure 6D-F). Collectively, these results indicate that MIF can enhance macrophage M2 polarization and impact the production of cytokines. The elevated soluble MIF further reinforces M2 polarization and cytokine secretion, contributing to a cascade of reactions involved in the recurrent mechanisms of CRSwNP as shown in the graphical abstract.

Discussion

The pathological mechanisms of recurrent CRSwNP are poorly clarified, the interactions of immune cells and the dysregulated expression of corresponding cytokines were demonstrated to play a pivotal role in mediating postoperative recurrence [22, 25]. Considering this, outlining the serum cytokine profiles offers a valuable opportunity to gain insights into the systemic immune status of CRSwNP patients and their vulnerabilities to postoperative recurrence. Recently, the application of multiple cytokine analyses as a novel tool has gained increasing attention in comprehending the inflammatory changes linked to airway inflammatory diseases [26, 27]. Through the analysis of cytokine profiles, prior studies have identified several cytokines that are closely associated with the disease severity and prognosis in asthma and allergic rhinitis patients [27, 28]. To date, few studies have utilized multiple serum cytokine analyses to explore objective biomarkers for predicting postoperative recurrence and discovering the underlying recurrent mechanisms of CRSwNP. In this prospective study, we first analyzed serum levels for 48 cytokines in a discovery cohort. Our findings revealed a distinctive baseline serum cytokine profile in recurrent CRSwNP when compared to non-recurrent cases. The candidate cytokines were subsequently validated in an independent cohort, confirming

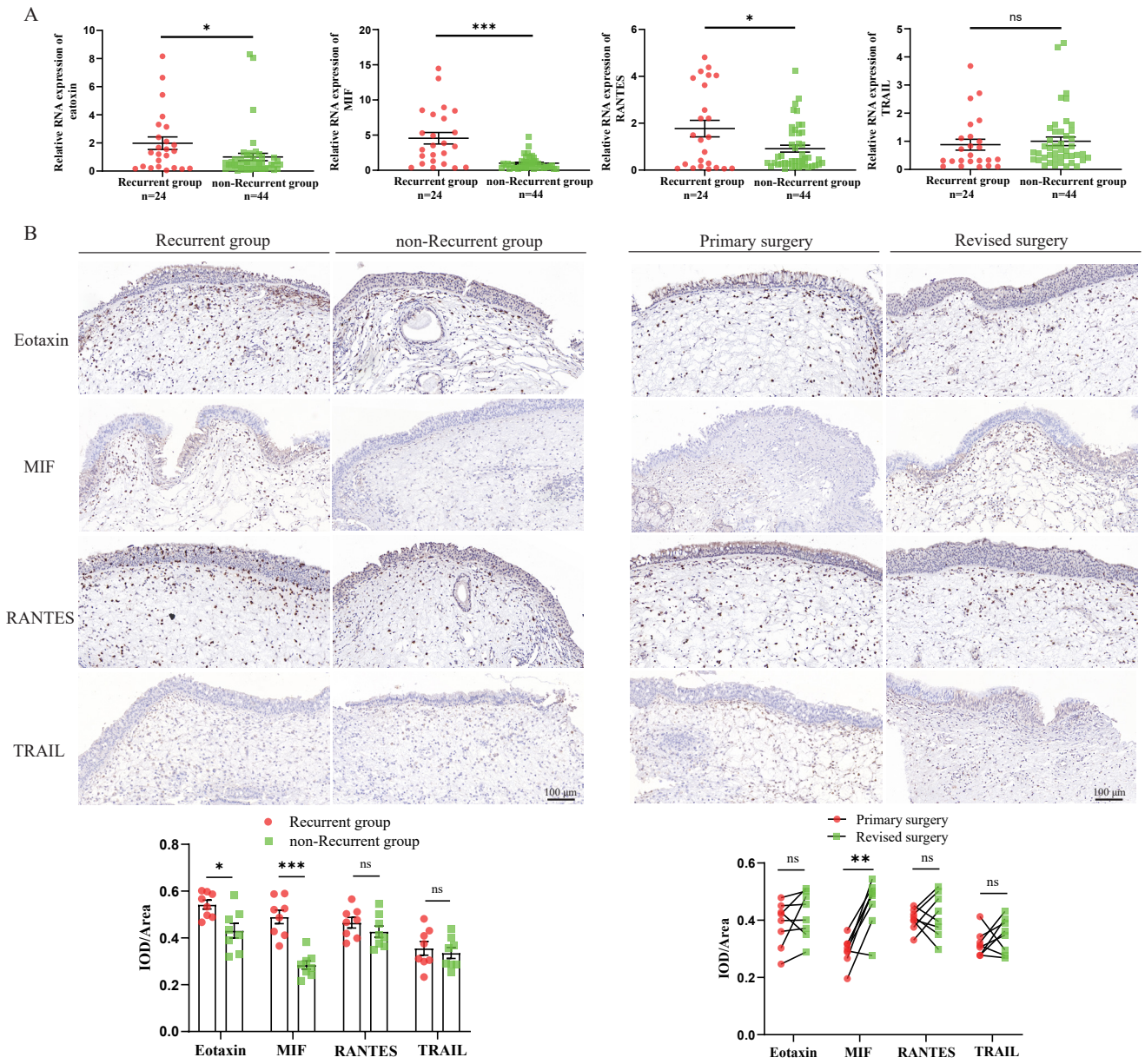

Figure 3. Tissue cytokine expressions and their associations with postoperative recurrence in CRSwNP patients. (A) the comparisons of tissue eotaxin, MIF, RANTES, and TRAIL mRNA expressions between recurrent and non-recurrent groups; (B) IHC staining exploring the tissue levels of eotaxin, MIF, RANTES, and TRAIL between recurrent and non-recurrent groups; (C) IHC staining evaluating the tissue levels of eotaxin, MIF, RANTES, and TRAIL between primary surgery and revised surgery groups. CRSwNP, chronic rhinosinusitis with nasal polyps; IHC, immunohistochemistry.

the predictive values of serum eotaxin, MIF, RANTES, and TRAIL for postoperative recurrence in CRSwNP. Furthermore, tissue expressions of eotaxin, MIF, and RANTES were found to be elevated in recurrent CRSwNP, with tissue MIF demonstrating a positive correlation with serum MIF levels. Intriguingly, MIF levels were significantly higher in nasal polyp tissues when patients experienced postoperative recurrence in comparison to their baseline levels. These results suggest that elevated MIF is closely involved in the mechanisms underlying the recurrence of CRSwNP.

MIF is a molecule with multiple biological activities, and it is highly expressed in various immune cells, particularly macrophages [29, 30]. Previous studies reported that MIF was essential in the development and differentiation of macrophages, and elevated MIF promoted macrophage polarization and cytokine secretions, which contributed to the pathogenesis of various autoimmune diseases, malignant tumors, and inflammatory diseases [31, 32]. However, its role in nasal inflammatory diseases and the underlying mechanisms are poorly understood. Recently, we found that serum MIF levels were significantly elevated in allergic rhinitis patients and correlated with disease severity, and serum MIF concentrations could potentially serve as an indicator of immune tolerance status and be linked to

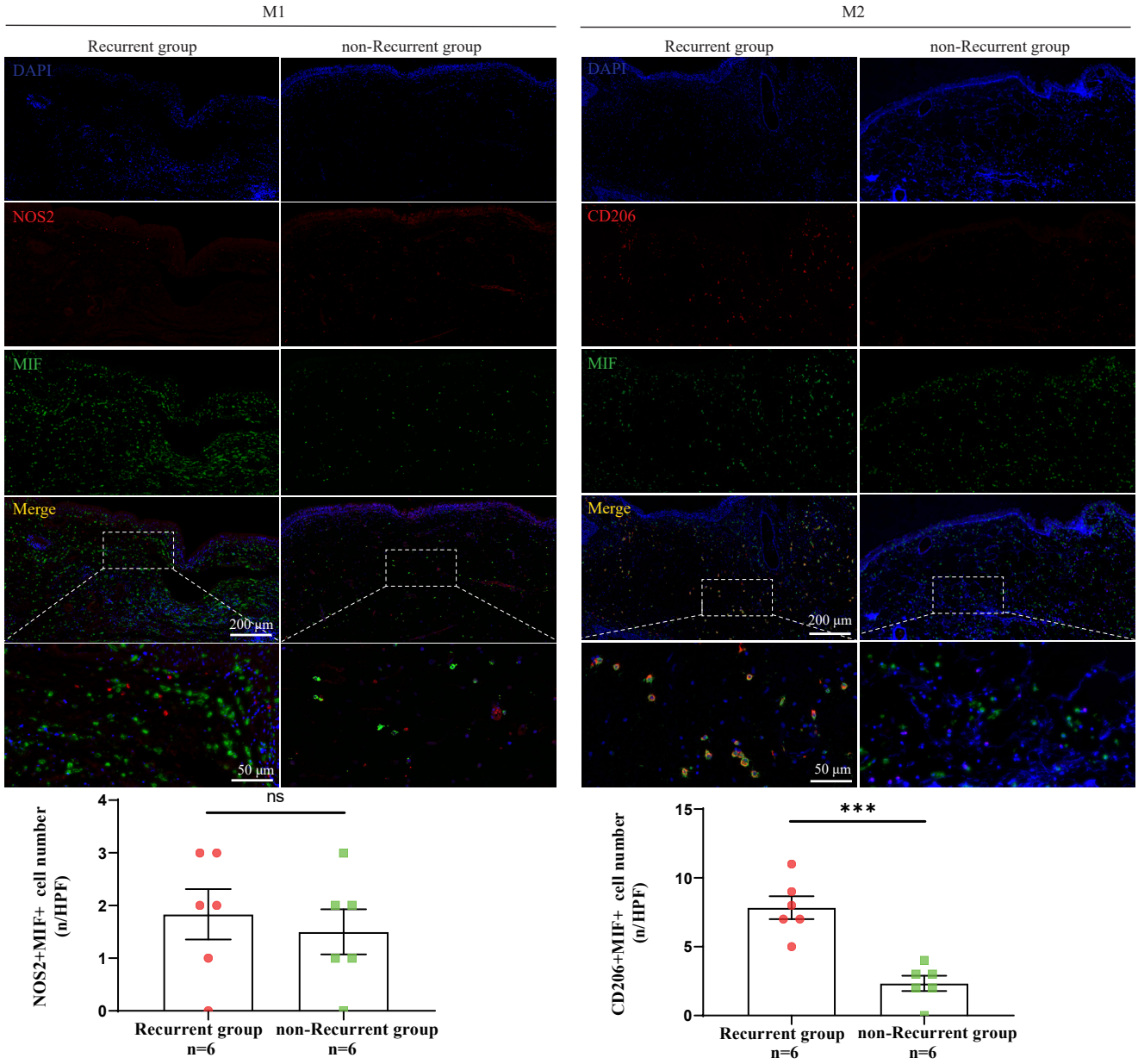

Figure 4. Multiplex immunofluorescence staining detecting the co-expressions of MIF and macrophage polarization markers in recurrent and non-recurrent CRSwNP patients. CRSwNP, chronic rhinosinusitis with nasal polyps.

the efficacy of sublingual immunotherapy [17, 28]. Furthermore, our previous study observed that both serum and tissue MIF were increased in CRSwNP patients in comparison with CRSsNP patients and HC, and tissue MIF expression was correlated with the degree of eosinophilic inflammation [17]. These results suggest that the altered expression of MIF could potentially impact macrophage function and play a role in shaping the immunological microenvironment within the nasal mucosa. However, its influence on postoperative recurrence and underlying effects are not clarified.

In the present study, we observed that MIF was increased in the recurrent CRSwNP, and tissue MIF was co-expressed with CD206. The in vitro experiments confirmed that the overexpression of MIF stimulated macrophage M2 polarization and the production of inflammatory cytokines and soluble MIF. Additionally, exogenous MIF also promoted macrophage M2 polarization and the secretion of TGF-β1 and CCL-24. It was widely recognized that eosinophil infiltration, the excessive accumulation of Th2-type cytokines, and TGF-β1-mediating tissue remodeling were the primary pathogenic mechanisms that contribute to tissue heterogeneity in recurrent CRSwNP [33, 34]. Remarkably, macrophage M2 polarization not only intensified tissue remodeling through the release of inflammatory mediators but also enhanced eosinophilic inflammation by attracting and facilitating the

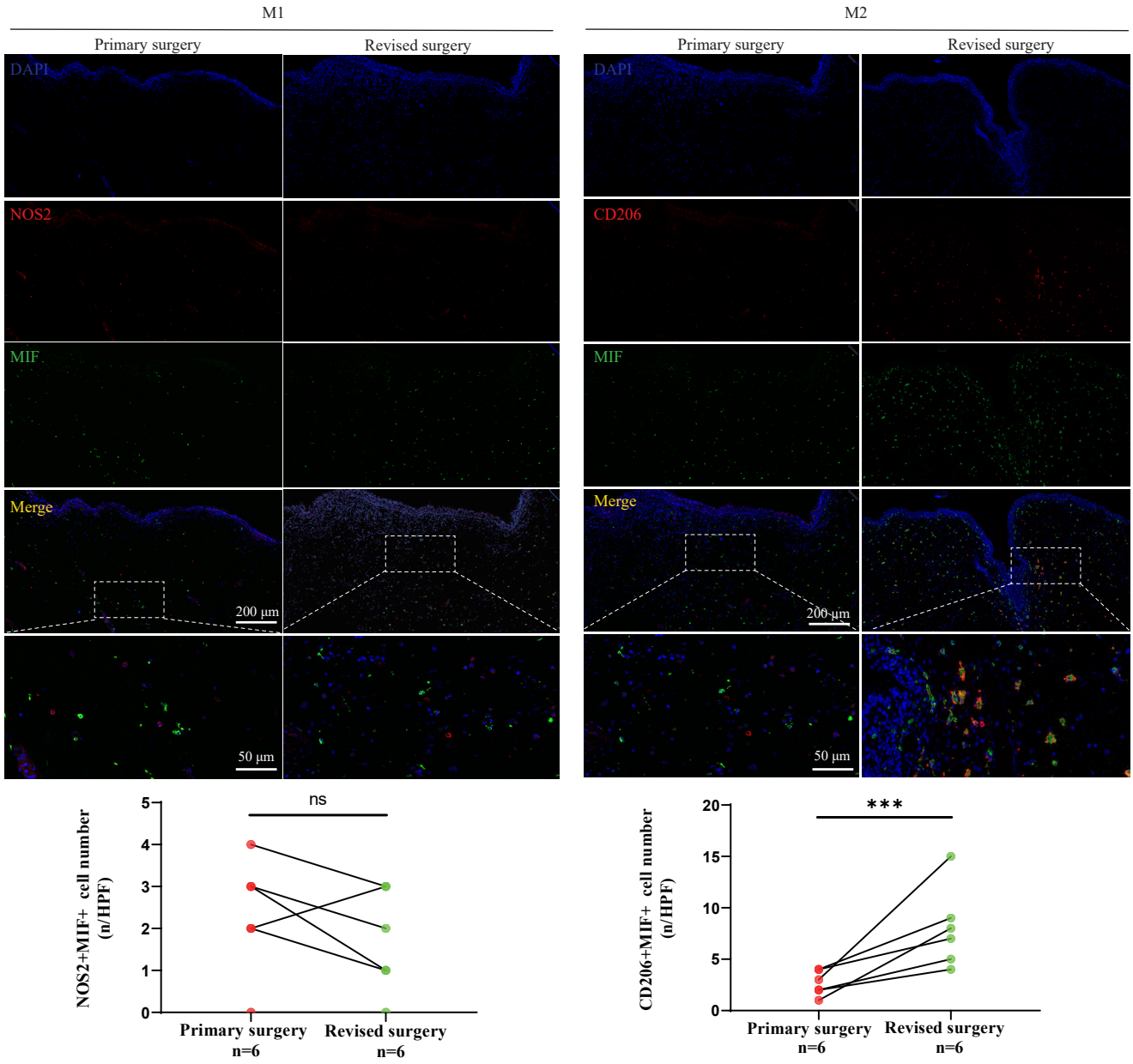

Figure 5. Multiplex immunofluorescence staining of MIF and macrophage polarization markers in tissue collected during primary and revised surgery from the same CRSwNP patients. CRSwNP, chronic rhinosinusitis with nasal polyps.

migration of eosinophils into the tissues [35]. Emerging evidence suggested that MIF was a crucial upstream regulator of innate immunity and inflammation [36, 37]. Excessive MIF expression has been proven to be associated with heightened inflammation and immunopathology via regulating macrophage polarization [38]. Previous studies revealed that MIF, primarily produced by macrophages and monocytes, intensified the activation of immune cells and the production of pro-inflammatory cytokines, particularly in the context of type 2-mediated inflammation and eosinophilic inflammation [39, 40]. Experimental evidence demonstrated that MIF promoted macrophage M2 polarization and the secretion of chemokines, including CCL-24, subsequently facilitating the recruitment of eosinophils to the inflammatory site, which exacerbated the eosinophilic inflammation at the inflammatory tissues and impacted disease prognosis [41, 42]. Collectively, we propose a hypothesis that external stimuli and allergens initiate the nasal mucosa, triggering phagocytosis by macrophages and inducing the intracellular expression of MIF, promoting the polarization of M2 macrophages and the release of soluble MIF. The heightened soluble MIF further reinforces M2 polarization, and cytokine secretion, and triggers a cascade of reactions, ultimately resulting in increased levels of TGF-β1 and CCL24. These cytokines impact mucosal epithelial cells and recruit eosinophils into nasal mucosa, leading to tissue damage,

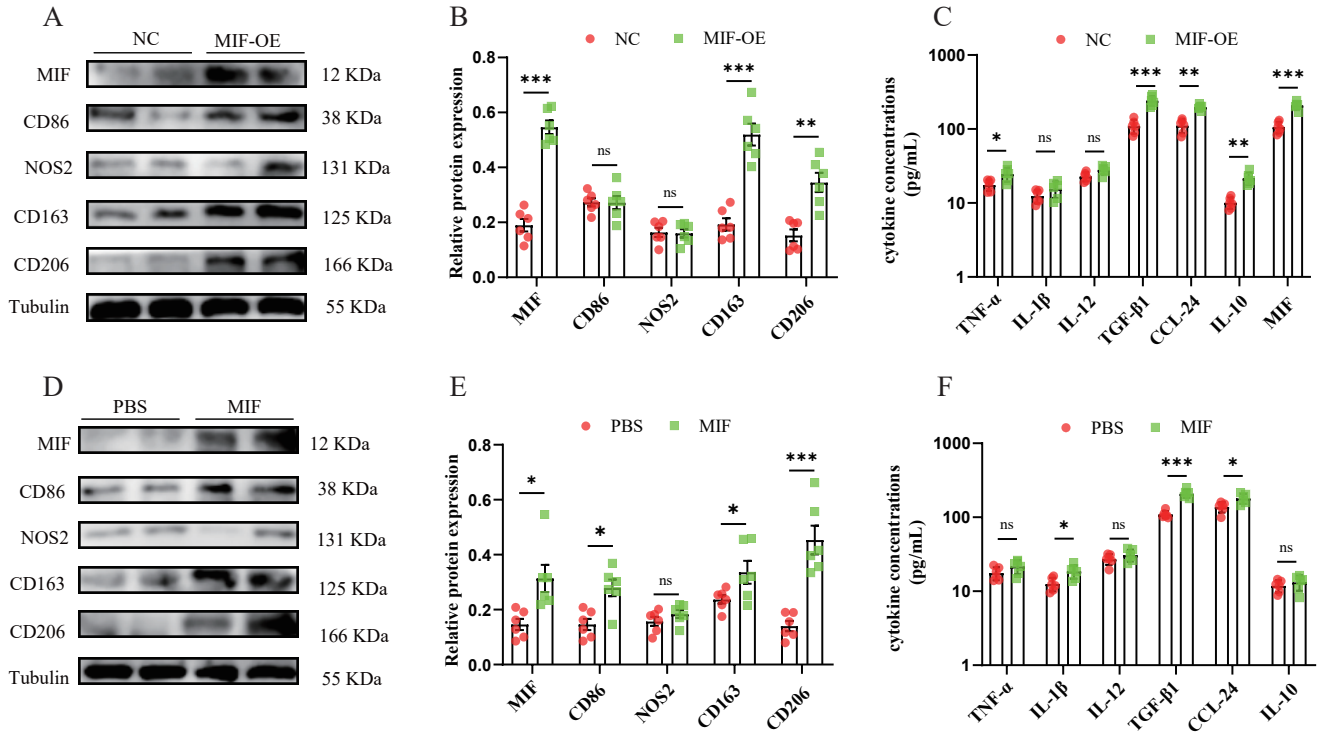

Figure 6. MIF facilitates macrophage M2 polarization and cytokine secretion in vitro. (A-C) Overexpression of MIF enhanced the expressions of CD163 and CD206 in macrophages and induced the secretion of TNF-α, TGF-β1, CCL-24, IL-10, and MIF in cell supernatant. (A) representative blots of target proteins; (B) densitometric analysis of proteins; (C) cytokines detected by ELISA. (D-F) MIF recombinant protein (20 ng/mL) increased CD163 and CD206 expressions in macrophages and promoted the production of IL-1β, TGF-β1, and CCL-24 in cell supernatant. *P<0.05, **P<0.01, ***P<0.001, ns, no significance.

remodeling, and eosinophilia, thus contributing to the recurrence of CRSwNP (graphical abstract).

Another interesting finding was that recurrent CRSwNP patients exhibited higher baseline serum and tissue concentrations of eotaxin, and serum TRAIL in comparison with non-recurrent patients, and serum eotaxin and TRAIL presented potential values for predicting postoperative recurrence. Eotaxin is a crucial chemokine with a potent chemoattractant effect on eosinophils, and elevated serum eotaxin concentrations may facilitate the recruitment of eosinophils to the site of inflammation, thus exacerbating and sustaining eosinophilic inflammation, playing a significant role in the pathogenesis and prognosis of various inflammatory diseases [43]. Moreover, higher levels of eotaxin may impact tissue repair following inflammation, exacerbating mucosal fibrosis and tissue remodeling, affecting the therapeutic efficacy and prognosis of airway inflammatory diseases [44, 45]. Previous research has identified the upregulation of multiple family members of eotaxin in CRSwNP tissues, and the expression levels of eotaxin-2 and eotaxin-3 correlate positively with tissue eosinophilic inflammation [46, 47]. TRAIL is a member of the TNF superfamily with a crucial role in immune response and cell apoptosis [48, 49]. Elevated concentrations of TRAIL not only promote the activation of immune cells and the recruitment of eosinophils but also induce apoptosis in mucosal epithelial cells, affecting mucosal repair, exacerbating tissue remodeling, and influencing the development and outcome of airway inflammatory diseases [50]. Therefore, we have reason to believe that elevated eotaxin and TRAIL concentrations may be involved in the pathological mechanisms of postoperative recurrence by promoting the recruitment of eosinophils to the nasal mucosa and tissue remodeling.

The present study has several limitations. First, this is a single-center prospective study with the same ethnicity and limited sample size, which may raise the risk of selection bias and affect the generalization. Second, we only analyzed baseline cytokine levels in CRSwNP patients and their association with postoperative recurrence; we did not analyze differences in cytokine expression between CRSwNP patients and HC. Third, we conducted only relatively simple cell regulation experiments and did not further discover the in-depth mechanisms through animal model experiments.

Conclusion

In this prospective study, we presented an innovative method to predict postoperative recurrence in CRSwNP using serum analysis of multiple cytokines. Our findings revealed that distinc-

tive serum cytokine profiles were linked to the risk of postoperative recurrence in CRSwNP patients. The discovery-validation results suggested that MIF could potentially serve as a reliable biomarker for predicting postoperative recurrence in CRSwNP. Mechanistically, MIF promoted macrophage M2 polarization and cytokine secretion, setting in motion a cascade of reactions that contributed to the recurrent mechanisms of CRSwNP.

Authors' contributions

SX wrote the paper. ZT, ZX, JZ, and CY performed research, analyzed data, and designed the research. WJ and HZ designed and supervised the research. All authors critically read the paper and approved the manuscript.

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Conflicts of interest

There are no patents, products in development, or marketed products to declare. Authors of this manuscript have no relevant financial or other relationships to disclose.

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SUPPLEMENTARY MATERIAL

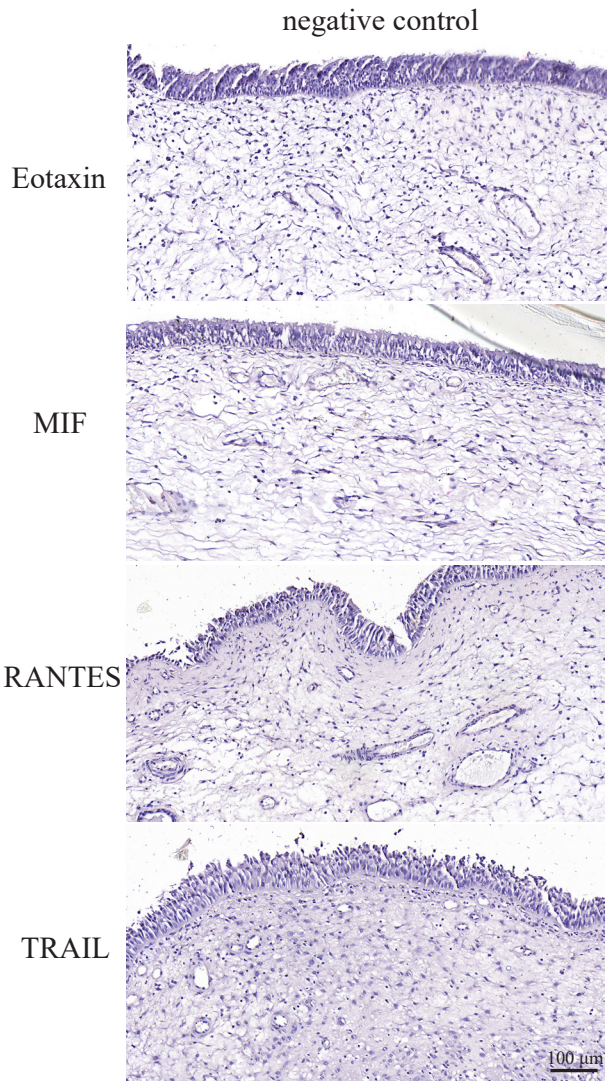

Figure S1. The negative controls of eotaxin, MIF, RANTES, and TRAIL in tissue IHC. IHC: immunohistochemistry.

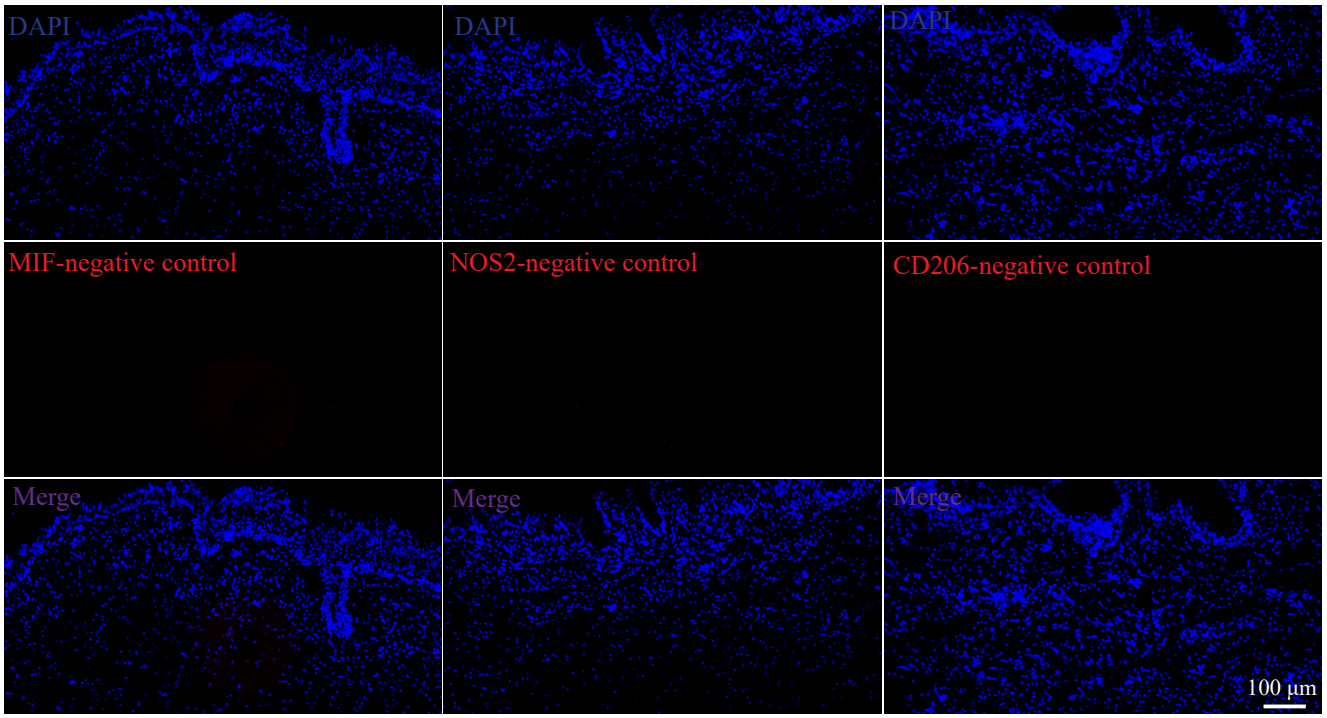

Figure S2. The negative controls of MIF, NOS2, and CD206 in tissue immunofluorescence.

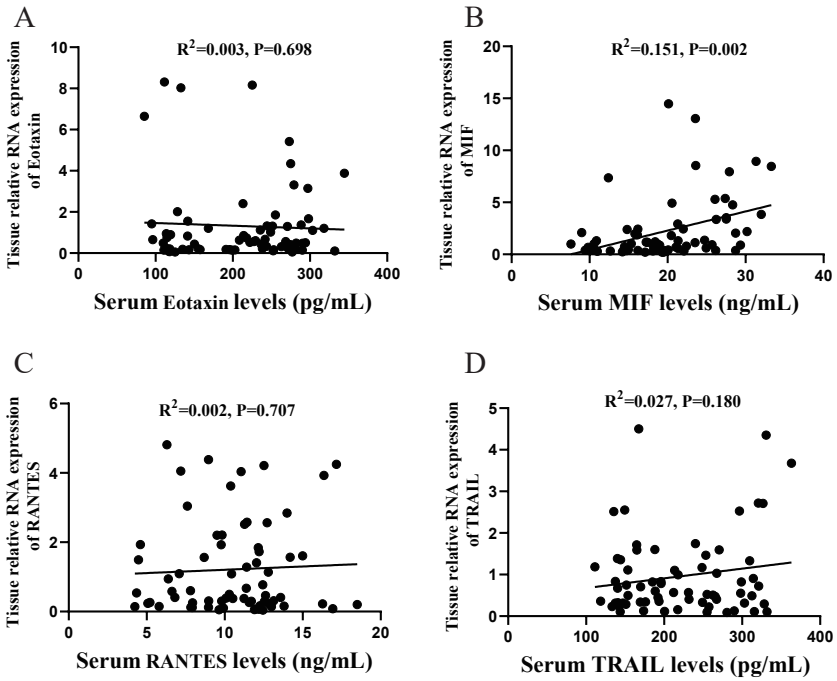

Figure S3. The correlations between tissue expressions of eotaxin (A), MIF (B), RANTES (C), and TRAIL (D) and their serum levels in CRSwNP patients. CRSwNP, chronic rhinosinusitis with nasal polyps.

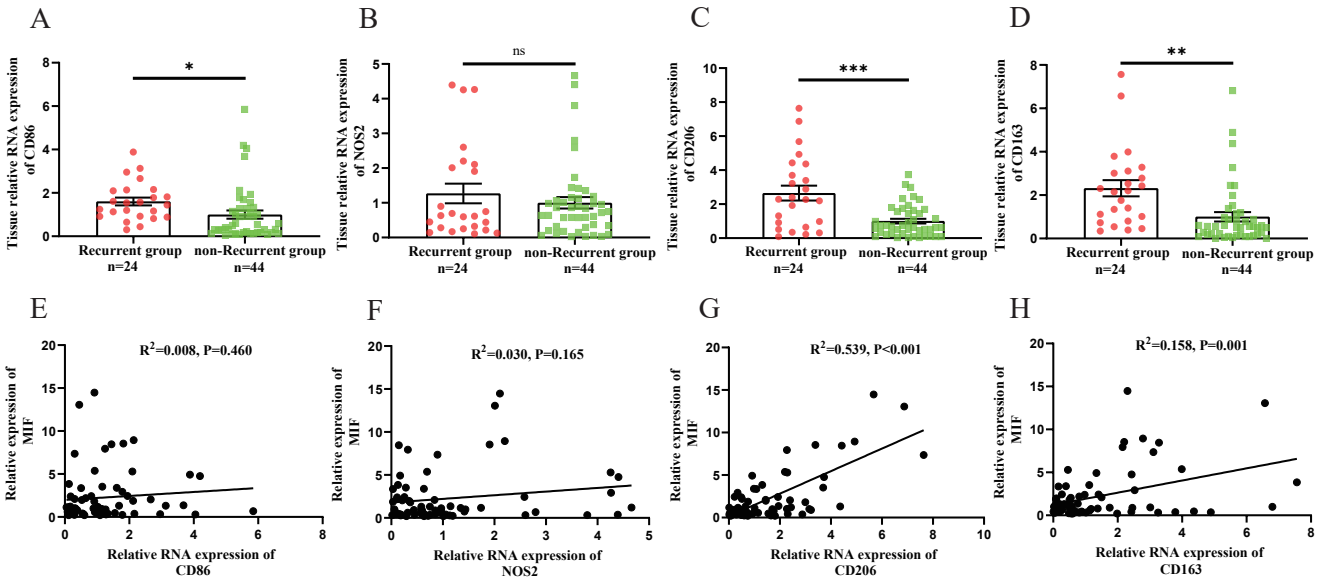

Figure S4. Tissue expressions of M1 and M2 polarization markers in recurrent and non-recurrent CRSwNP patients and their associations with tissue MIF levels. CRSwNP, chronic rhinosinusitis with nasal polyps. (A-D) comparisons of tissue expressions of M1 and M2 polarization markers between recurrent and non-recurrent groups. (E-G) the correlations between tissue levels of MIF and M1 and M2 polarization markers in CRSwNP patients. CRSwNP, chronic rhinosinusitis with nasal polyps.

Table S1. Serum 48 cytokines, abbreviations, and their detection limit (pg/mL).

Cytokines	Abbreviation	Detection limit
Basic fibroblast growth factor	Basic FGF	13.3-17330
beta-Nerve growth factor	β-NGF	0.62-3950
Cutaneous T cell attracting chemokine	CTACK	1.3-14902
Eotaxin	Eotaxin	0.06-3672
Granulocyte colony-stimulating factor	G-CSF	3.08-234050
Granulocyte-macrophage colony-stimulating factor	GM-CSF	0.34-16294
Growth-regulated oncogene alpha	GRO-α	27.06-88356
Hepatocyte growth factor	HGF	1.31-226440
Interferon alpha-2	IFN-α2	0.42-222378
Interferon gamma	IFN-γ	0.6-4632
Interleukin-10	IL-10	0.52-31536
Interleukin-12(p40)	IL-12(p40)	4.46-146618
Interleukin-12(p70)	IL-12(p70)	0.88-103398
Interleukin-13	IL-13	0.13-14748
Interleukin-15	IL-15	51.94-420160
Interleukin-16	IL-16	0.48-60976
Interleukin-17	IL-17	0.88-112216
Interleukin-18	IL-18	0.44-32684
Interleukin-1 alpha	IL-1α	1.58-51724
Interleukin-1beta	IL-1β	0.12-9340
Interleukin-1 receptor antagonist	IL-1ra	29.44-150288

Cytokines	Abbreviation	Detection limit
Interleukin-2	IL-2	0.72-58186
Interleukin-2R alpha	IL-2R α	1.3-41144
Interleukin-3	IL-3	0.06-3492
Interleukin-4	IL-4	0.1-10404
Interleukin-5	IL-5	3.6-136870
Interleukin-6	IL-6	0.28-8160
Interleukin-7	IL-7	0.92-54966
Interleukin-8	IL-8	0.22-31100
Interleukin-9	IL-9	0.72-47820
Interferon-inducible protein 10	IP-10	0.52-31536
Leukemia inhibitory factor	LIF	2.38-141266
Monocyte chemotactic protein 1	MCP-1	0.56-16084
Monocyte chemotactic protein 3	MCP-3	0.46-2454
Macrophage colony-stimulating factor	M-CSF	0.34-31854
Macrophage migration inhibitory factor	MIF	1.48-83270
Monokine induced by Interferon-gamma	MIG	0.68-59010
Macrophage inflammatory protein-1 alpha	MIP-1α	0.08-1826.77
Macrophage inflammatory protein-1 beta	MIP-1β	0.44-7678
Platelet-derived growth factor-BB	PDGF-BB	2.46-56248
tumor necrosis factor-related apoptosis-inducing ligand	RANTES	1.56-12432
Stem cell factor	SCF	0.64-68710
Stem cell growth factor- beta	SCGF-β	74.8-3477188
Stromal cell-derived factor-1 alpha	SDF-1α	1.4-58836
Tumor necrosis factor-alpha	TNF-α	1.88-159898
Tumor necrosis factor- beta	TNF-β	0.74-33522
Tumor necrosis factor-related apoptosis-inducing ligand	TRAIL	0.26-12682
Vascular endothelial cell growth factor	VEGF	10.32-72442

Table S2. Primer sequence of human genes.

Gene	Forward primer	Reverse primer
Eotaxin	TGGGAATGGGGAATGGCTCTG	TCCCCCTGGGACCTCGTTCTTCTCT
MIF	AAGCTGCTGTGCGGCCTGCT	AGCCCACATTGGCCGCGTTC
RANTES	GGCAACTGATGCTTCCCAACGTCA	TGTGGGGAGGCTTCCAAAGCTCA
TRAIL	CCCCCTCCCAAATGACTTGGCTGT	CACAGTGTCCTTCCCTGCCTCCCTTA
CD86	CTGTAACTCCAGCTCTGCTCCGTA	GCCCATAAGTGTGCTCTGAAGTGA
NOS2	CTGGAGGTCAATTCCTGGAAAA	TCCCCGTTTCCTTCCTGACAGCAG
CD163	GCCATAGTGAGTGTGGGCACAA	TCAGTGTGGCTCAGAATGGCCTC
CD206	GCCCGGAGTCAGATCACACA	AGTGGCTCAACCCGATATGACAG
GAPDH	CATGAGAAGTATGACAACAGCCT	AGTCCTTCCACGATACCAAAGT