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Engulfment and cell motility protein 1 fosters reprogramming of tumor-associated macrophages in colorectal cancer

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Abstract

Functional reprogramming of tumor-associated macrophages (TAMs) is crucial to their potent tumor-supportive capacity. However, the molecular mechanism behind the reprogramming process remains poorly understood. Here, we identify engulfment and cell motility protein 1 (ELMO1) as a crucial player for TAM reprogramming in colorectal cancer (CRC). The expression of ELMO1 in stromal but not epithelial tumor cells was positively associated with advanced clinical stage and poor disease-free survival in CRC. An increase in ELMO1 expression was specifically found in TAMs, but not in other multiple nonmalignant stromal cells. Gain- and loss-of-function assays indicated ELMO1 reprogrammed macrophages to a TAM-like phenotype through Rac1 activation. In turn, ELMO1-reprogrammed macrophages were shown to not only facilitate the malignant behaviors of CRC cells but exhibited potent phagocytosis of tumor cells. Taken together, our work underscores the importance of ELMO1 in determining functional reprogramming of TAMs and could provide new insights on potential therapeutic strategies against CRC.

KEYWORDS
colorectal cancer, ELMO1, TAM reprogramming, tumor-associated macrophage

1 | INTRODUCTION

According to global cancer statistics in 2021, colorectal cancer (CRC) ranks third in incidence and cancer-related mortality, and causes approximately 149,500 new cases and 52,980 deaths worldwide each year.[1] The clinical outcomes of CRC patients at early or locally advanced stage have been significantly improved by current therapeutic methods.[2] Unfortunately, there is enormous difficulty in treating patients with metastatic CRC; this group of patients has a median survival time of less than 2 years and a 5-year survival rate of less than 15%.[3–5] These facets point to an unmet requirement for more effective therapeutic approaches for CRC patients.

As a major component of the tumor microenvironment, tumor-associated macrophages (TAMs) comprise up to approximately 50% of the tumor mass, including CRC.[6] Tumor-associated macrophages in CRC are primarily a macrophage subpopulation with M2-like phenotype, and have the potential to reduce antitumor immunity and facilitate tumor progression.[7–9] Preclinical studies have confirmed targeting TAMs could offer the promise of improved cancer outcomes, and clinical trials with therapeutic agents that combat TAMs are currently underway.[6,10] These insights highlight the importance of TAMs in CRC. However, knowledge of the underlying mechanism of TAM reprogramming in the tumor-promoting phenotype remains insufficient. Thus, further understanding the reprogramming and the contribution of TAMs in CRC pathogenesis will help to develop alternative treatment options and improve clinical outcomes.

Since its discovery in 2001, engulfment and cell motility protein 1 (ELMO1), as a member of the engulfment and cell motility protein family, had been originally shown to dictate phagocytic capacity in both nematodes and humans.[11,12] Subsequent studies found ELMO1 has a significant association with several cancer-related pathways, such as nuclear factor-κB (NF-κB),[13] Rac activation,[14] and Dock2 signaling.[15] In agreement, gain- and loss-of-function experiments indicated ELMO1 has the potential to promote malignant biological properties in certain cancers, such as human glioma,[16] hepatocellular carcinoma,[17,18] and ovarian cancer.[19] These insights show that ELMO1 could represent an orchestrator of tumor pathogenesis. Moreover, recent evidence suggests ELMO1 plays vital roles in disorders, especially those with macrophages involved in pathogenesis.[20–22] However, the significance of ELMO1 in CRC pathogenesis remains an important knowledge gap. Considering the importance of macrophages in CRC, we enquired whether ELMO1 contributes to TAM-based support for tumor progression. Accordingly, we documented TAMs to determine the contribution of ELMO1 to CRC, and further characterize the significance of ELMO1-reprogrammed macrophages in tumor malignancy.

2 | MATERIALS AND METHODS

2.1 | Patients and tissue samples

Fresh tumor tissues and paired normal adjacent samples were collected from 12 patients with CRC from October 2021 to December 2021. Formalin-fixed, paraffin-embedded (FFPE) whole-tumor tissues were collected from 150 CRC patients from January 2020 to December 2021 who received radical surgery at the Sixth Affiliated Hospital of Sun Yat-sen University. All specimens were pathologically confirmed adenocarcinoma. The procedures for specimen collections were carried out with the approval of the Institutional Review Board of the Sixth Affiliated Hospital of Sun Yat-sen University (Register Number: E2021093). The human peripheral blood monocytes (PBMs) were collected from 12 CRC patients between October 2021 and December 2021 with the approval of the Institutional Review Board of the Sixth Affiliated Hospital of Sun Yat-sen University (Register Number: E2021093). Informed written consent was obtained from all subjects.

2.2 | Quantitative real-time RT-PCR

Total RNA was extracted from cells or tissue samples using TRIzol Reagent (Thermo Fisher Scientific, Cat#15596026) based on the manufacturer's recommendations. Quantitative real-time RT-PCR (qRT-PCR) was carried out using SYBR Green PCR Master Mix (Applied Biosystems, Cat#4472937) on the Applied Biosystems 7500 Sequence Detection system using the SYBR Green detection protocol as outlined by the manufacturer. All reactions were performed in a 10 μl reaction volume in triplicate. Standard curves were generated and the relative amount of target gene mRNA was calculated using the $2^{-\triangle\triangle CT}$ method with normalization to 18S. The primer sequences (BGI) used in the present study are listed as follows:

GAPDH forward, 5′-GAGAAGGCTGGGGCTCATTT-3′ and reverse, 5′- AGTGATGGCATGGACTGTGG -3′; and ELMO1 forward, 5′-TCCTGAAAATCCGCCAGTCC -3′ and reverse, 5′-CAGG GTGTCCAGGTCATTCC -3′.

2.3 | Western blot analysis

Western blotting was undertaken using anti-ELMO1 (1:1000; Abcam, Cat#ab155775), anti-CD163 (1:1000; Abcam, Cat#ab182422), anti-HLA-DR (1:1000; Abcam, Cat#ab20181), anti-inducible nitric oxide synthase (iNOS) (1:5000; Abcam, Cat#ab178945), anti-GAPDH (1:1000; CST, Cat#5174 s), anti-CD68 (1:1000; Abcam, Cat#ab283654), anti-α-smooth muscle actin (1:1000; CST, Cat#19245 s), anti-CD31 (1:1000; CST, Cat#3528 s), and anti-CD3 (1:1000; Abcam, Cat#ab16669) Abs (4°C overnight). Secondary Abs (1:5000; Abcam, Cat#ab205718, #ab6789) were incubated for 1 h at room temperature. The images were cropped for presentation based on the molecular mass marker proteins. Signals were developed with ECL Blotting Detection Reagents (Santa Cruz Biotechnology, Cat#SC-2048) and specific protein bands were visualized on X-ray film. Some of the protein bands were analyzed by grayscale value in ImageJ (version 1.8).

2.4 | Immunohistochemistry

Immunohistochemistry (IHC) was carried out on slides of FFPE whole-tumor tissues and the primary Abs used were anti-CD163

(1:100; Abcam, Cat#ab182422), and anti-ELMO1 (1:100; Abcam, Cat# ab155775). The slides were incubated with primary Abs at 4°C overnight, appropriate secondary Abs, and then stained with 3,3'-diaminobenzidine complex and counterstained with hematoxylin. The density of TAMs per patient was determined by counting the number of $CD163^+$ immune cells per tissue sample.[8,23] For the expression of ELMO1 in epithelial tumor and stromal cells, images of ELMO1 IHC expression were processed using a manual segmentation of tumor and stroma compartments in a 20× magnification field according to previous descriptions.[24] Then the staining intensity of ELMO1 expression in tumor and stroma compartments was graded as four stages: 0, none; 1, weak; 2, moderate; and 3, strong; The staining proportion score was counted from 1% to 100%. The final histoscore was quantified by proportion score × intensity score.

2.5 | Isolation of primary cells from tumors and paired normal adjacent tissues

Single-cell suspensions from human tumor tissues were generated using the Tumor Dissociation Kit (Miltenyi Biotec, Cat#130–095-929). Dissociation of paired normal adjacent tissues was carried out on the basis of previous description.[25] Using a Dead Cell Removal Kit (Miltenyi Biotec, Cat#130–090-101), dead cells were eliminated. Lymphocytes, macrophages, fibroblasts, endothelial cells, and primary tumor cells were then purified with $CD3^+$ microbeads (Miltenyi Biotec, Cat#130–050-101), $CD14^+$ microbeads (Miltenyi Biotec, Cat#130–050-201), Anti-Fibroblast MicroBeads (Miltenyi Biotec, Cat#130–050-601), $CD31^+$ microbeads (Miltenyi Biotec, Cat#130–091-935), and epithelial cell adhesion molecule $(EpCAM)^+$ microbeads (Miltenyi Biotec, Cat#130–061-101), respectively.

2.6 | Monocyte isolation and macrophage culture

Human PBMs from healthy volunteer donors were isolated by density-gradient centrifugation using Ficoll–Hypaque (Pharmacia, Cat# IAE-1). Harvested PBMs were seeded at a density of 2×10^6 cells/well per 24-well plate in DMEM (Gibco, Cat#12430062) supplemented with 10% heat-inactivated human AB serum (Gemini Bio-Products, Cat#100–512), 50 U of penicillin per ml, 50 μg of streptomycin per ml, 2 mM L-glutamine, and 20 ng/ml human macrophage colony-stimulating factor (R&D Systems, Cat#416-ML) to stimulate macrophage differentiation. After 6 days of culture, nonadherent cells were removed by repeated gentle washing with a warm medium, and more than 95% of the adherent cells generated from current procedures were $CD14^+$ monocytes/macrophages, indicating a good purity of monocytes/macrophages. For in vitro activation, monocyte-derived macrophages at 2×10^6 cells were treated for 1 day with 45 ng/ml recombinant human interleukin-4 (IL-4; R&D Systems, Cat#204-IL) for M2 polarization cell models or 25 μg/ml lipopolysaccharide (LPS; Sigma, Cat#LPS25) in order to generate M1 polarization cell models.

2.7 | Cell transduction

Transduction of ELMO1-specific siRNAs was undertaken using an siRNA pool (Merck). The ELMO1-specific siRNAs or nontargeting control siRNAs were transfected into the indicated cells that were plated at 5×10^5 cells per ml in 6-well plates, transfected with 5 nmol/ml specific siRNA duplexes with 3 μl/ml Lipofectamine 3000 (Invitrogen, Cat#L3000001), and cocultured with Opti-MEM (Gibco, Cat#12430062, NYC) for 6 h. The detailed information was on the basis of the manufacturer's recommendations. For the Elmo1 overexpression in macrophages, cells were plated at 5×10^5 cells per ml in 24-well plates and transduced with lentiviral particles (MOI 100; Guangzhou Bioyard Biotechnology Development Co., Ltd) with 5 μg/ml Polybrene (Sigma, Cat# TR-1003) according to previous description.[26]

2.8 | Cell culture and in vitro function assays

Human CRC cell lines SW480 and DLD1 were purchased from ATCC. Dulbecco's modified Eagle's medium was used to culture cells with 10% FBS as well as 1% penicillin–streptomycin. Cells were incubated at 37°C under a humidified atmosphere containing 5% CO_2. For plate colony formation assay, 500 SW480 or DLD1 cells with the indicated treatment were seeded into 6-well plates and cultured at 5% CO_2 at 37°C for 2 weeks. The cells were then fixed with methanol and stained with 1% crystal violet for 15 min at room temperature. The colony numbers were calculated using ImageJ software. For cell migration and invasion assays, cells were plated in a 24-well plate using an 8 μm pore size chamber (Corning, Cat#3422) without Matrigel (Corning, Cat#356234) (for migration assays) or with Matrigel (for invasion assays). SW480 or DLD1 cells (5×10^4) with 200 μl serum-free cell culture media were placed into the upper chamber, while culture medium supplemented with 10% FBS was placed in the lower chamber. After incubating at 37°C for 48 h, the cells at the lower surface of the filters were fixed in methanol for 10 min and stained with crystal violet for 10 min. The cells were then imaged and counted.

2.9 | Enzyme-linked immunosorbent assay

After treatment of the macrophages, the cell supernatant was obtained and centrifuged at 5000 g for 5 min at 4°C. Tumor necrosis factor-α, IL-6, IL-10, and transforming growth factor-β levels were measured in duplicate using the ELISA kit (R&D Systems, Cat#DTA00D, #QK206, #D1000B, #DY240) according to the manufacturer's instructions.

2.10 | Determination of phagocytic ability of macrophages against tumor cells

The indicated tumor cells predyed by CellTracker CTDR (Thermo Fisher Scientific, Cat# C34565) were cocultured with macrophages predyed by CellTracker CMFDA (Thermo Fisher Scientific, Cat# C7025). Macrophages labeled by CMFDA were coincubated with tumor cells labeled by CTDR in a 1:1 (4×10^5:4×10^5) ratio for 24 h. Macrophages were then rigorously washed by PBS, digested by 10× TrypLE Selected Enzyme (Gibco, Cat# A1217701) diluted in 5× PBS with 1 mM EDTA and subjected for flow cytometry analysis. Double-positive cells ($CMFDA^+$ $CTDR^+$) represent the macrophages that engulfed tumor cells.

2.11 | Statistical analysis

All data are shown as mean ± SD unless otherwise indicated, and the SPSS 16.0 (IBM) statistical package was adopted for all the statistical analyses. To test the statistical significance, two-tailed Student's *t*-test or one-way ANOVA was applied for continuous variables with normal distributions, whereas the Mann–Whitney or Kruskal–Wallis test was introduced if distributions were skewed. The criterion for statistical significance was a p value of less than 0.05.

3 | RESULTS

3.1 | Expression of ELMO1 in epithelial tumor cells of CRC tissues

To probe the contribution of ELMO1 to CRC pathogenesis, we examined the mRNA and protein expression of ELMO1 in fresh tumor tissues and paired normal adjacent samples isolated from a set of 12 patients with CRC. Determined by qRT-PCR, ELMO1 mRNA was 3.19 ± 1.16-fold higher in the whole-tumor samples than their corresponding normal tissues (Figure 1A). Likewise, western blotting indicated a comparable expression pattern for ELMO1 protein (Figure 1B). Furthermore, IHC for ELMO1 expression in whole-tumor tissues from another set of 150 patients with stage I–III CRC indicated ELMO1-positive cells were observed in both the epithelial tumor and stromal cells (Figure 1C). However, no significant differences were found for epithelial tumor cell expression of ELMO1 (tumoral ELMO1 hereafter) according to TNM stages (Figure 1D). Next, two categories were stratified for tumoral ELMO1 using a cut-off point of the median expression levels (ELMO1 IHC score 1.65). The results showed that patients with high versus low levels of tumoral ELMO1 had a similar risk of recurrence (Figure 1E). Likewise, patients with high versus low levels of tumoral ELMO1 were shown to have a comparable 3-year disease-free survival (DFS) (Figure 1F). Collectively, these results indicated that tumoral ELMO1 does not seem to correlate with CRC progression.

3.2 | Clinical implication of ELMO1 expression in stromal cells of CRC tissues

As tumoral ELMO1 expression did not associate with CRC progression, we next sought to gain insights into the clinical significance of ELMO1 expression in stromal cells (stromal ELMO1 hereafter) of CRC tissues. There was no significant correlation between the tumoral and stromal ELMO1 (Figure 2A). When correlating stromal ELMO1 with clinicopathologic status, stromal ELMO1 was highly abundant in advanced TNM stages (Figure 2B), but there was a weak association of stromal ELMO1 with KRAS status (Figure 2C). Stromal ELMO1 was then stratified into two categories (high vs. low) using a cut-off point of the median expression (ELMO1 IHC score 1.6) levels. Results indicated that patients with elevated levels of stromal ELMO1 had a higher recurrence risk, which showed recurrence at 3 years was identified in 21 (28%) versus 9 (12%) patients with high versus low levels of stromal ELMO1 (Figure 2D). Patients with high versus low levels of stromal ELMO1 had a shorter DFS (hazard ratio 2.46; 95% confidence interval, 1.2–5.0; $p = 0.02$). Three-year DFS was recorded for 54 (72%) versus 66 (88%) patients with high versus low levels of stromal ELMO1 (Figure 2E). Therefore, these findings suggested a significant contribution of stromal ELMO1 to CRC progression.

3.3 | Expression of ELMO1 is upregulated in TAMs

As the tumor stroma consists of multiple nonmalignant stromal cells, including lymphocytes, macrophages, endothelial cells, and fibroblasts, we further investigated from which type of cells the stromal ELMO1 participates in tumor progression. To address this issue, stromal cells were isolated from fresh CRC tissues and paired normal adjacent samples. These cells were verified by western blot analysis through their specific Abs (Figure 3A–D). Notably, western blotting showed that macrophages isolated from fresh tumor tissues were found to have a significant increase of ELMO1 expression in comparison with those isolated from paired normal adjacent samples (Figure 3E,F), whereas other stromal cells, including lymphocytes, endothelial cells, or fibroblasts, showed no significant differences in ELMO1 expression (Figure 3G–L). Together, these data suggested that TAMs appeared to mediate the contribution of stromal ELMO1 to CRC progression.

3.4 | Engulfment and cell motility protein 1 reprograms macrophages to a TAM-like phenotype

As shown above, ELMO1 expression in TAMs is likely to correlate with CRC progression. We further confirmed that the upregulated expression of ELMO1 in IL-4-treated monocyte-derived macrophages (MDMs), but not LPS-treated MDMs (Figure 4A). For this, we set out to test the functional effects of macrophage

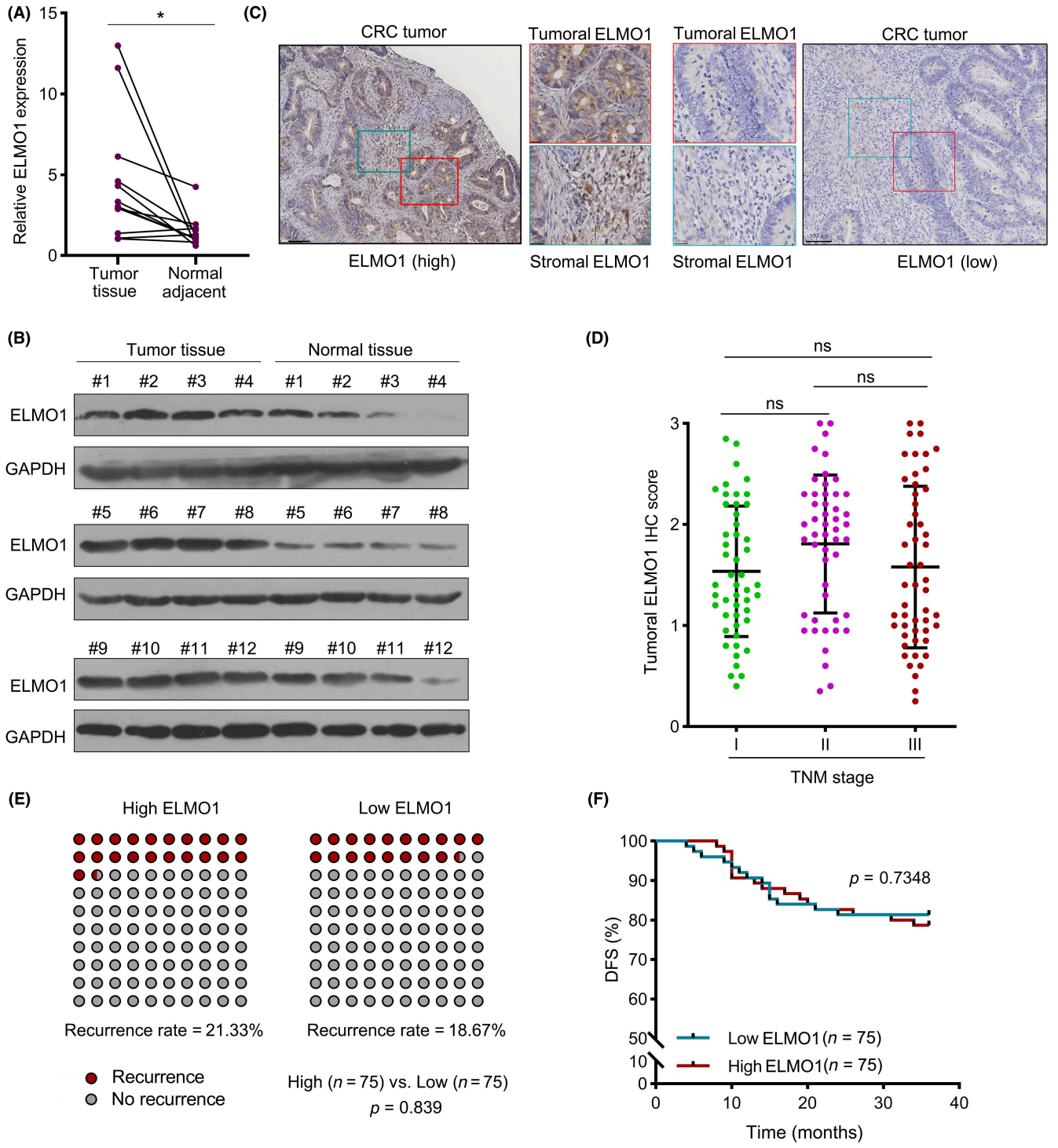

FIGURE 1 Tumoral engulfment and cell motility protein 1 (ELMO1) did not correlate with colorectal cancer (CRC) progression. (A) Expression of ELMO1 in CRC tumor tissues and normal adjacent tissues by quantitative real-time RT-PCR. *$p \leq 0.05$, Student's *t*-test. (B) Protein levels of ELMO1 in CRC tumor tissues and normal adjacent tissues by western blot analysis. (C) ELMO1 expression levels in CRC tumor tissues by immunohistochemistry (IHC). Scale bars, 100 and 25 μm. (D) Tumoral ELMO1 expression among different TNM stages. ns, $p > 0.05$ by one-way ANOVA. (E) Recurrence rate and (F) 3-year disease-free survival (DFS) in CRC patients with high versus low ELMO1 levels in tumoral cells. Statistical significance was assessed using the χ^2-test (E) and log–rank (Mantel–Cox) test (F). All values are mean ± SD.

ELMO1. Using serial sections of 16 CRC tissues stained for ELMO1 and the TAM marker CD163, we found that stromal ELMO1 was positively associated with the TAM density (Figure 4B,C), whereas there seemed to be little association between tumoral ELMO1 and the TAM density (Figure 4C, left panel). To determine the contribution of ELMO1 to macrophage reprogramming, we

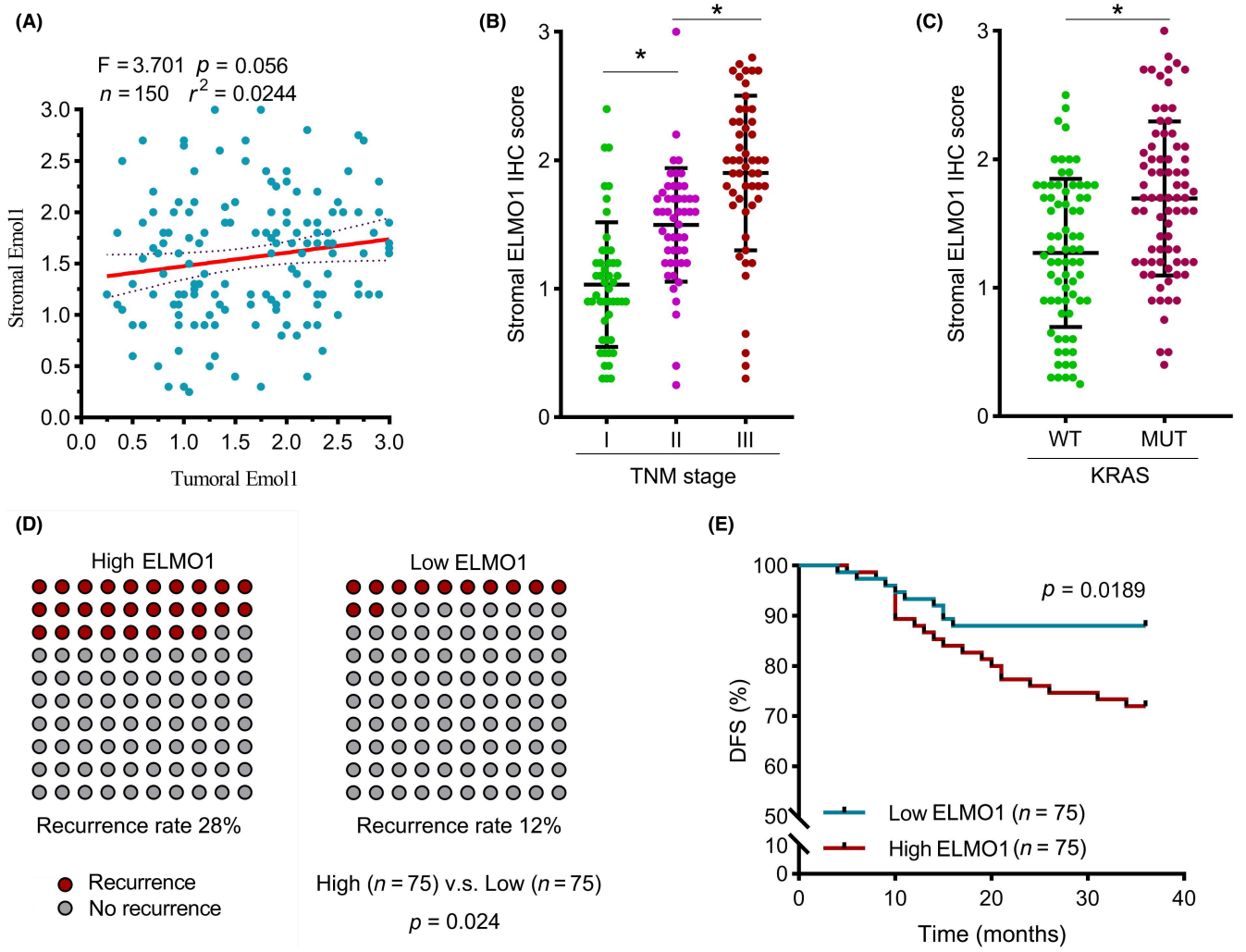

FIGURE 2 Engulfment and cell motility protein 1 (ELMO1) expression in stromal cells of colorectal cancer (CRC) tissues and clinical implications. (A) Association between tumoral ELMO1 and stromal ELMO1 in CRC tissue. Statistical significance was assessed using simple linear regression. (B) Stromal ELMO1 expression among different TNM stages. *$p \leq 0.05$, one-way ANOVA. (C) Stromal ELMO1 expression in CRC tissues with WT versus mutant (MUT) KRAS. *$p \leq 0.05$, Student's *t*-test. (D) Recurrence rate and (E) 3-year disease-free survival (DFS) in CRC patients with high versus low ELMO1 levels of stromal cells. Statistical significance was assessed using the χ^2-test (D) and log–rank (Mantel–Cox) test (E). All values are mean ± SD. IHC, immunohistochemistry.

overexpressed ELMO1 in MDMs (Figure 4D, left panel), followed by IL-4 treatment. Overexpression of ELMO1 led to a significant increase in IL4-induced M2-phenotype markers (Figure 4E,F), but no marked differences in the expression of M1-phenotype markers for the LPS-triggered M1 polarization cell models (Figure 4G). Furthermore, an ELMO1-specific siRNA pool was used to knock down ELMO1 expression (Figure 4D, right panel) in TAMs from CRC patients. The reduction of ELMO1 in TAMs substantially decreased the expression of TAM marker CD163, and slightly increased the expression of M1 markers (HLA-DR and iNOS) (Figure 4H). As expected, ELMO1 expression was tightly associated with the ability to produce tumor-supportive cytokines of TAMs (Figure 4I,J). Together, these results revealed that ELMO1 had the ability to reprogram macrophages to a TAM-like phenotype.

3.5 | Rac1 activation contributes to ELMO1-reprogrammed macrophages

Given the well-established link of ELMO1 with Rac1 activation,[27,28] we therefore explored the involvement of Rac1 activation in ELMO1-reprogrammed macrophages. As anticipated, macrophages from fresh CRC tissues versus those from paired normal adjacent samples had a dramatical increase in Rac1 activation (Figure 5A). More importantly, treating TAMs with the Rac1 inhibitor NSC23766 effectively abrogated the TAM-like phenotype, as indicated by the substantial reduction in CD163 expression and production of tumor-supportive cytokines (Figure 5B,C). To further confirm the role of Rac1 activation, IL-4-induced MDM M2 polarization cell models were introduced. Results showed that Rac1 activation was shown to be significantly increased in ELMO1-overexpressed MDMs (Figure 5D), and inhibition

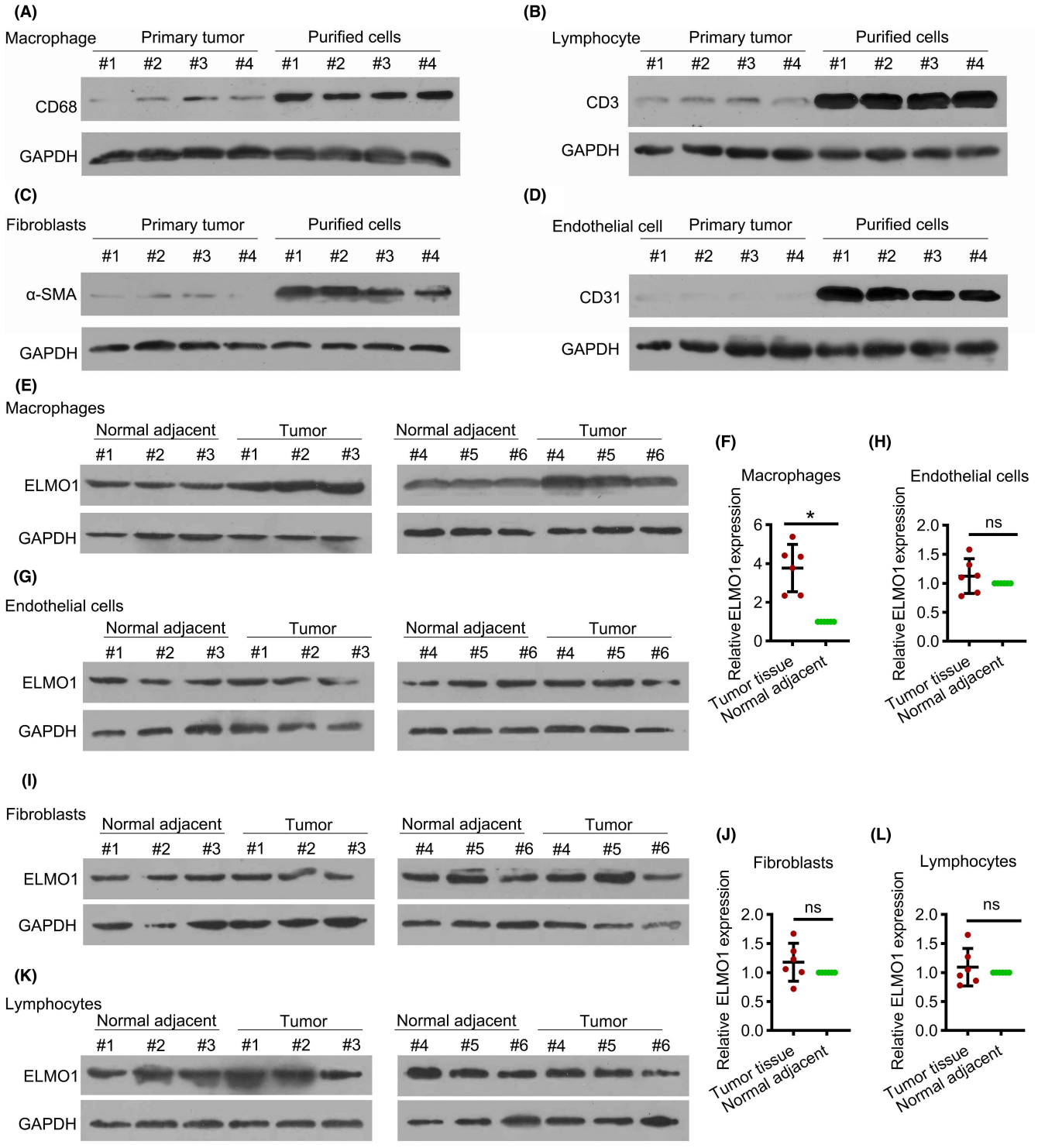

FIGURE 3 Engulfment and cell motility protein 1 (ELMO1) expression is upregulated in tumor-associated macrophages. (A–D) Isolated cells were verified by their specific markers. (E, F) ELMO1 expression in macrophages isolated from fresh colorectal cancer (CRC) tissue. (G, H) ELMO1 expression in endothelial cells isolated from fresh CRC tissue. (I, J) ELMO1 expression in fibroblasts isolated from fresh CRC tissue. (K, L) ELMO1 expression in lymphocytes isolated from fresh CRC tissue. *$p \leq 0.05$, Student's *t*-test. α-SMA, α-smooth muscle actin; ns, $p > 0.05$, Student's *t*-test. All values are mean ± SD.

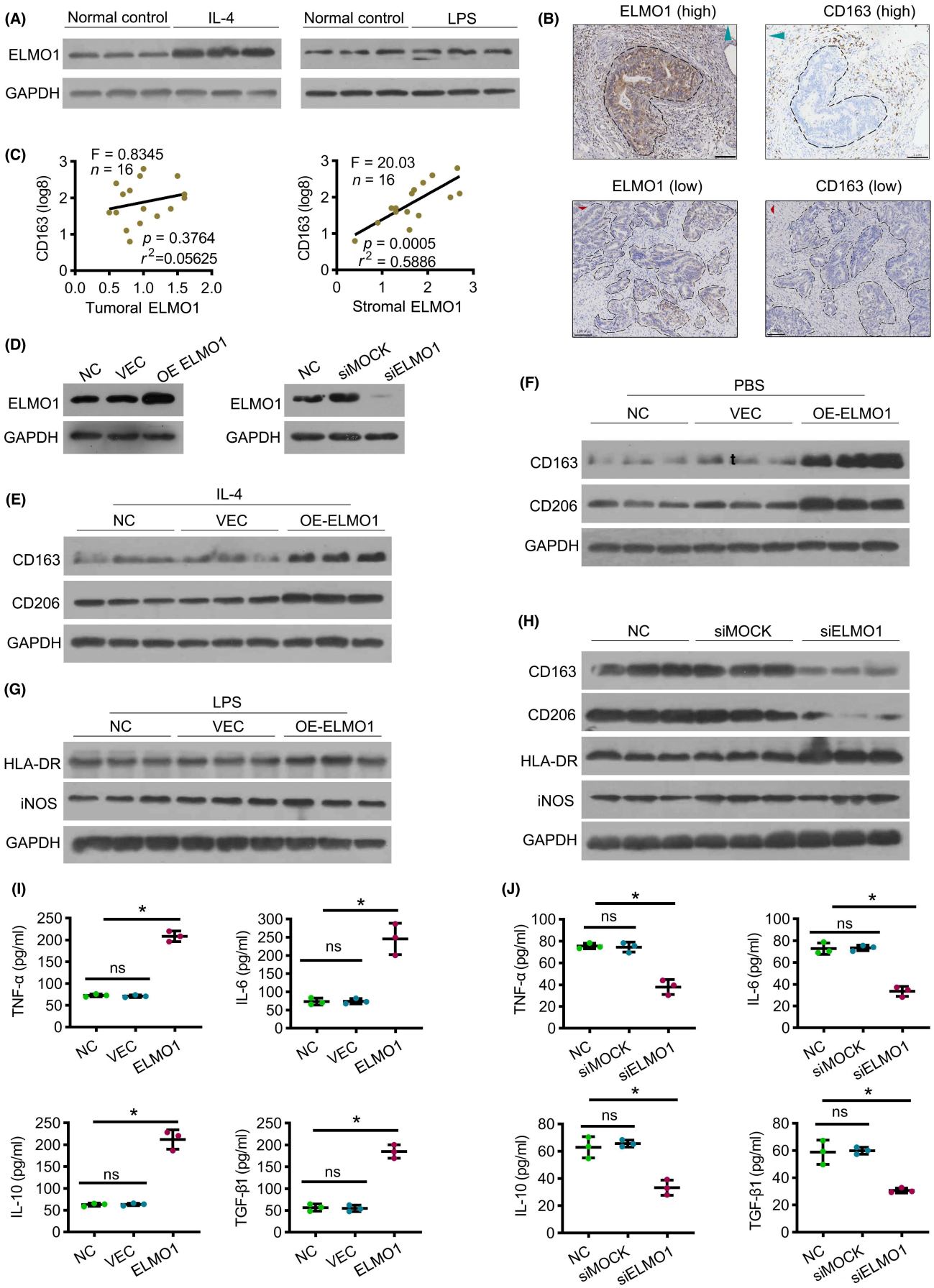
(A)
Normal control
IL-4
Normal control
LPS
ELMO1
GAPDH
(B)
ELMO1 (high)
CD163 (high)
ELMO1 (low)
CD163 (low)
(C)
F = 0.8345
n = 16
p = 0.3764
r2=0.05625
CD163 (log8)
Tumoral ELMO1
F = 20.03
n = 16
p = 0.0005
r2 = 0.5886
Stromal ELMO1
(D)
NC
VEC
OE ELMO1
siMOCK
siELMO1
(E)
IL-4
CD163
CD206
(F)
PBS
OE-ELMO1
(G)
LPS
HLA-DR
iNOS
(H)
(I)
TNF-α (pg/ml)
IL-6 (pg/ml)
IL-10 (pg/ml)
TGF-β1 (pg/ml)
ns
(J)

FIGURE 4 Engulfment and cell motility protein 1 (ELMO1) reprograms macrophages to a tumor-associated macrophage (TAM)-like phenotype. (A) Monocyte-derived macrophages (MDMs) from healthy donors were treated with interleukin-4 (IL-4) or lipopolysaccharide (LPS). Western blot analysis was used to detect ELMO1 expression after the indicated treatments. (B) Expression of CD163 and ELMO1 in colorectal cancer (CRC) tissues by immunohistochemistry. Scale bar, 100 μm. (C) Association between tumoral ELMO1, stromal ELMO1, and CD163 in CRC tissue. Statistical significance was assessed using simple linear regression. (D) Efficiency of lentiviral particle overexpression (OE) of ELMO1 and siRNA knockdown of ELMO1 were detected by western blot analysis. (E) MDMs from healthy donors were transfected with vector plasmid and ELMO1 plasmid by lentiviral particles, and then treated with IL-4. Western blot was used to detect CD163 and CD206 expression. (F) MDMs from healthy donors were transfected with vector plasmid (VEC) and ELMO1 plasmid by lentiviral particles. Western blot was used to detect CD163 and CD206 expression. (G) MDMs from healthy donors were transfected with vector plasmid and ELMO1 plasmid by lentiviral particles, and then treated with LPS. Western blot was used to detect HLA-DR and inducible nitric oxide synthase (iNOS) expression. (H) MDMs from healthy donors were transfected with siMOCK and siELMO1 RNA. Western blot was used to detect relative gene expression. Cytokines of TAMs were detected by ELISA after MDMs were transfected with ELMO1 (I) plasmid or (J) siRNA. Cell supernatant was obtained for detecting relative cytokines. $^{*}p \leq 0.05$, one-way ANOVA. NC, negative control; ns, $p > 0.05$, one-way ANOVA; TGF-β1, transforming growth factor-β1; TNF-α, tumor necrosis factor-α. All values are mean ± SD.

of Rac1 activation significantly suppressed the M2 transformation of macrophages (Figure 5E). Together, these data indicated Rac1 activation contributes to ELMO1-reprogrammed macrophages.

3.6 | Macrophage ELMO1 promotes tumor progression

Subsequently, we investigated whether inhibition of macrophage reprogramming through targeting ELMO1 could combat tumors. To this end, MDMs were transfected with the ELMO1-specific siRNA pool, followed by IL-4 induction. SW480 and DLD CRC cells were cocultured with these cells in Transwell plates for 7 days and then collected for further experiments. After coculture with IL-4-induced MDM M2 polarization cell models, the ability of CRC cells to grow, migrate, and invade was significantly increased. By contrast, inhibition of ELMO1 in IL-4-induced MDM M2 polarization cell models significantly abrogated their tumor-supportive capacity (Figure 6A–C). To confirm these findings, TAMs and autologous primary tumor cells were isolated from fresh CRC samples, and then were predyed by CellTracker CMFDA Dye and CellTracker CTDR Dye, respectively. Indeed, a significant ability of phagocytosis of tumor cells was observed in TAMs, whereas inhibition of ELMO1 resulted in a substantial increase in phagocytic activity of TAMs against the corresponding primary tumor cells (Figure 6D). All these results gave us a hint that macrophage ELMO1 played vital roles in promoting tumor progression.

4 | DISCUSSION

This study provides important insights into the critical role of ELMO1 in determining functional reprogramming of TAMs in CRC. Our findings showed an increase in the expression of ELMO1 in TAMs of CRC patients, and that high ELMO1 levels in TAMs indicated poor patient survival. Mechanistically, ELMO1 reprogrammed macrophages to a TAM-like phenotype through Rac1 activation, which subsequently promoted CRC progression. The present work, to our knowledge, is the first study that comprehensively elucidates the role and targeted therapeutic potential of macrophage ELMO1 in CRC.

Engulfment and cell motility protein 1 has been reported to play a vital role in multiple diseases, including inflammatory bowel disease, osteoporosis, and various cancers.[29–32] Evidence has indicated the positive effects of ELMO1 on promoting cancer cell proliferation, invasion, and metastasis.[16,33,34] In order to understand the relationship between ELMO1 and CRC, we first detected the ELMO1 expression level in CRC tissue. The results showed ELMO1 was significantly enhanced in CRC tissue compared with normal adjacent control. Interestingly, we found tumoral ELMO1 has no relevance with TNM stage or 3-year DFS. By contrast, ELMO1 expression in stromal cells of CRC tissues was significantly associated with TNM stage and DFS of CRC patients. These results suggest ELMO1 might participate in the regulation of CRC progression potentially through affecting the tumor microenvironment.

It is well established that the tumor microenvironment has a profound impact on cancer cell functions. Moreover, cancer cells can interact with adjacent stromal cells in their microenvironment.[35] Although ELMO1 has been reported in various cancer cells, few researchers have focused on ELMO1 expression in stromal cells of the tumor microenvironment. The main stromal cells include immune cells, endothelial cells, and fibroblasts. All of these stromal cells are shown to play important roles in the regulation of cancer cells. Of note, immune cells have been considered as an important target in immune checkpoint blockade therapies.[36–38] To better understand the role of ELMO1 in stromal cells in CRC, multiple stromal cells were isolated from fresh CRC tissues and paired normal adjacent samples. By detecting the expression of ELMO1, this work identified that macrophages were the only stromal cells that presented different ELMO1 expression levels between tumor tissue and normal adjacent tissue. Thus, ELMO1 expression was significantly increased in TAMs. Therefore, these results indicate that high expression levels of ELMO1 potentially regulated TAMs to affect malignant cancer cells in CRC patients.

The functions of TAMs in CRC are fully confirmed. Colorectal cancer cells can cross-talk with macrophages in the tumor

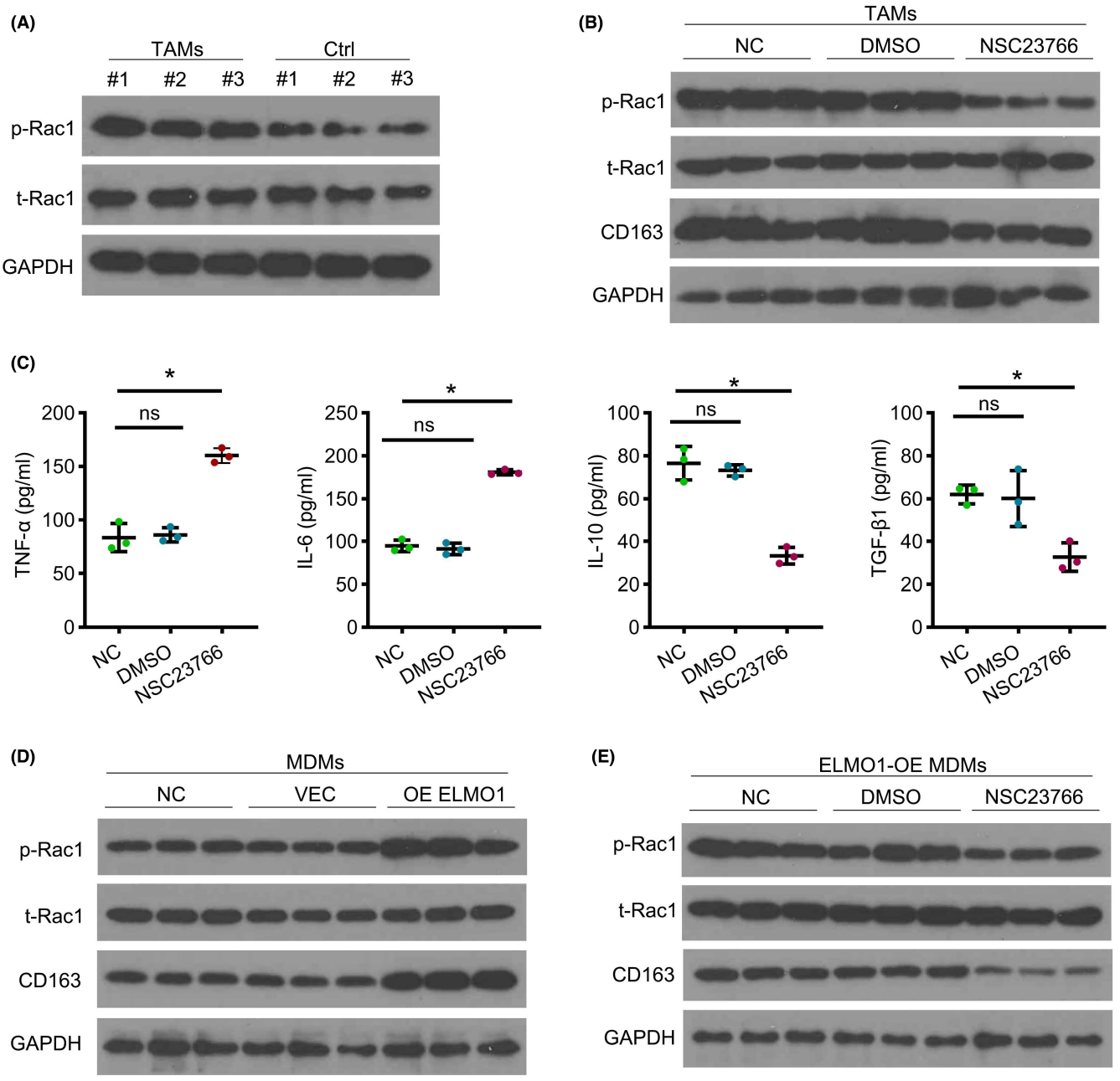

FIGURE 5 Rac1 activation contributes to engulfment and cell motility protein 1 (ELMO1)-reprogrammed macrophages. (A) Tumor-associated macrophages (TAMs) were isolated from fresh colorectal cancer (CRC) tissue and normal adjacent tissue; Rac1 expression is showed by western blot analysis. (B) TAMs were isolated from fresh CRC tissue then treated with Rac1 inhibitor NSC23766; western blot was used to detect relative gene expression. (C) ELISA was used to detect tumor-supportive cytokines of TAMs after NSC23766 treatment. *$p \leq 0.05$, one-way ANOVA. (D) Monocyte-derived macrophages (MDMs) were transfected with vector plasmid or ELMO1 plasmid, followed by western blot to detect relative gene expression. (E) MDMs were pretransfected with ELMO1, then treated with NSC23766. Western blot was used to detect relative gene expression. Ctrl, control; IL, interleukin; NC, negative control; ns, $p > 0.05$, one-way ANOVA; OE, overexpression; p-Rac, xxxx; TGF-β1, transforming growth factor-β1; TNF-α, tumor necrosis factor-α; t-Rac, xxxx. All values are mean ± SD.

microenvironment through exosomes and cytokines, which can induce TAMs to M2 polarization.[39,40] Tumor-associated macrophages in CRC are primarily a macrophage subpopulation with M2-like phenotype, and have the potential to reduce antitumor immunity and facilitate tumor progression.[7–9] To further confirm the ELMO1 function in TAMs, we analyzed the expression of CD163 and ELMO1 in CRC tumor samples. The results revealed these two markers were colocalized in CRC tumor tissue. More importantly, overexpression of ELMO1 in MDMs promoted M2-phenotype markers, but had no influence on M1-phenotype markers. In contrast, knockdown of ELMO1 could reverse the M2 polarization in TAMs from fresh CRC samples. Engulfment and cell motility protein 1 was relative with macrophage engulfment in apoptotic cell clearance and bacterial clearance,[21,22,41] which implied ELMO1

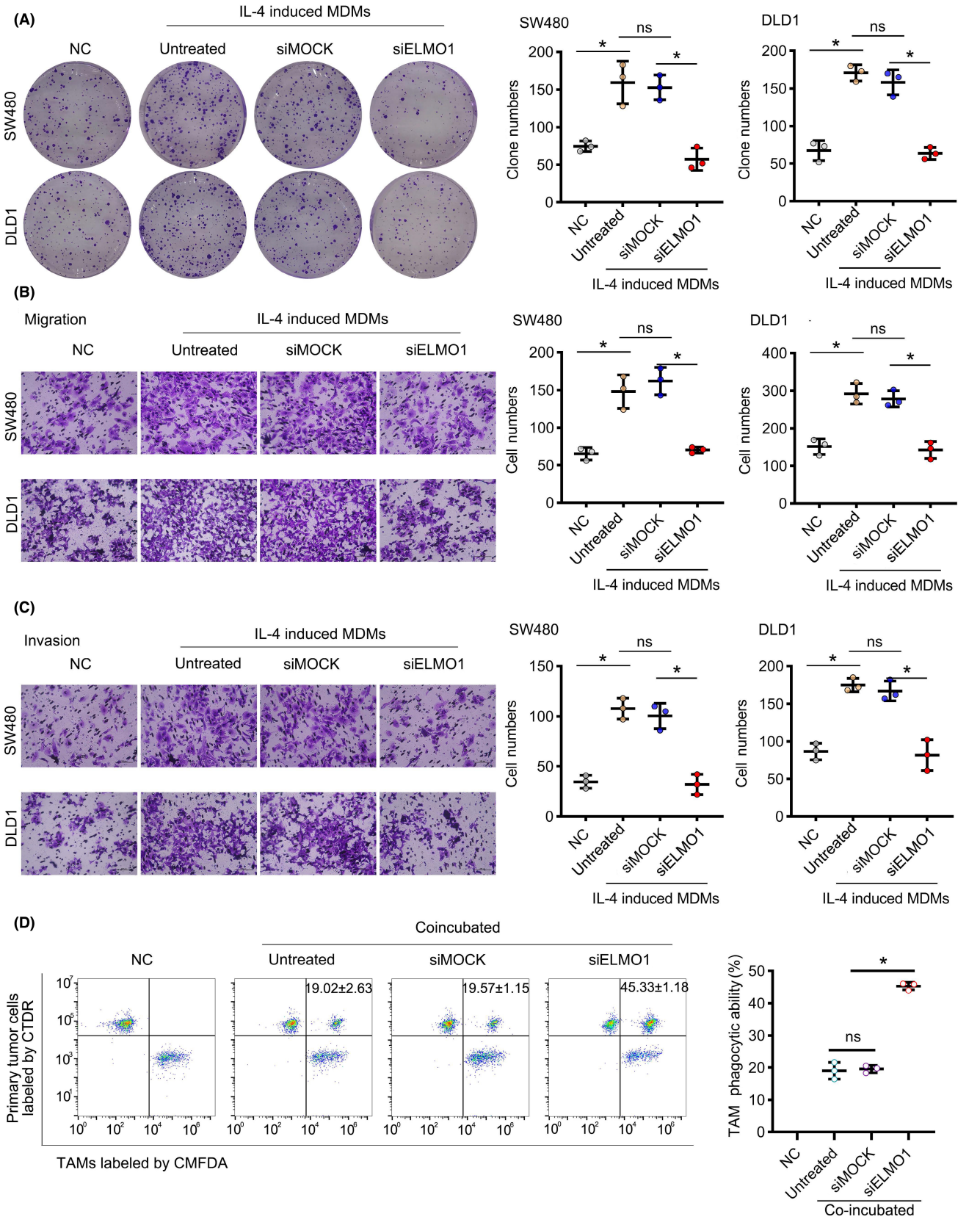

FIGURE 6 Macrophage engulfment and cell motility protein 1 (ELMO1) promotes tumor progression. (A–C) SW480 and DLD1 colorectal cancer cells were cocultured with monocyte-derived macrophages (MDMs) transfected with the ELMO1-specific siRNA pool, followed by interleukin-4 (IL-4) induction. SW480 and DLD1 cells were used for (A) clone formation, (B) Transwell for migration assay, and (C) Transwell for invasion assay. Scale bar, 100 μm. (D) Tumor-associated macrophages (TAMs) isolated from fresh CRC tissue were pretreated with siELMO1, then cocultured with autologous primary tumor cells. Phagocytic ability of TAMs was detected. *$p \leq 0.05$, one-way ANOVA. NC, negative control; ns, $p > 0.05$, one-way ANOVA. All values are mean ± SD.

expression in macrophages could enhanced the cell function under specific conditions. In this study, ELMO1 was enhanced in macrophages, which resulted in the TAM-like phenotype in CRC. The expression of M2-like macrophage markers CD163 and CD206 were increased under ELMO1 overexpression. Therefore, ELMO1 overexpression of macrophage results in the M2 macrophage phenotype switch in CRC, which is independent of related cytokine treatment and indicates that ELMO1 promotes progression in CRC. In addition, our data here showed the protumor function of ELMO1 in TAMs increased the production of tumor-supportive cytokines, and promoted the proliferation, migration, and invasion of CRC cells. Furthermore, targeting ELMO1 by siRNA can effectively abrogate the ability to produce tumor-supportive cytokines of TAMs and the tumor-promoting ability.

The present evidence indicates that Rac1 activation was associated with ELMO1 expression,[21,42,43] and ELMO1 is identified as a Rac1 regulator.[44] Rac1 is a small GTPase and it has been proven to participate in many dynamic cell biological processes like cell proliferation, cell motility, and cell invasiveness.[45] Rac1 expression is involved in intestinal homeostasis and Rac1 activation is enhanced in CRC.[45] A previous study reported that activation of the RhoA/Rho-associated protein kinase signaling pathway could significantly activate the anti-inflammatory M2 macrophage phenotype.[46] Additionally, PI3K-Akt-Rac1 signaling pathways were involved in insulin-induced macrophage phenotype switch (M2) and anti-inflammatory effects.[47] These studies indicated that Rac1 activation could promote M2 macrophage phenotype switch. Our data also confirmed these findings by showing that Rac1 activation significantly increased in TAMs from CRC samples. Inhibition of Rac1 reversed the M2 polarization in TAMs. In addition, the tumor-supportive cytokines were decreased in TAMs in response to Rac1 inhibitor NSC23766. Overexpression of ELMO1 significantly promoted Rac1 activation in macrophages, and this effect could be blocked by inhibition of Rac1. Together, these results indicated Rac1 activation contributed to ELMO1 in reprogramming macrophages in the tumor microenvironment.

The findings of this work could provide a novel therapeutic strategy by targeted correction of aberrant expression of ELMO1 in TAMs for CRC patients. However, there are issues requiring further efforts. First, as cancer cells can interact with stromal cells in the tumor microenvironment, it is not clear whether ELMO1 overexpression in TAMs is regulated by cancer cells. Second, ELMO1 is considered as an oncogene in various cancer cells; whether ELMO1 in TAMs could transfer to cancer cells needs further investigation. Finally, one active component of exosomes might take effect in different systems, and whether targeting ELMO1 could affect other organs and tissues needs to be determined.

In summary, this work indicates that ELMO1 participates in the pathogenesis of CRC through functional reprogramming of TAMs through Rac1 activation. Our results identify a new means by which ELMO1 affects tumor progression and provide new perspectives for therapeutic strategies for patients with CRC.

AUTHOR CONTRIBUTIONS

BW, SL, LR, YPY, ZWZ, and SSL performed study design. BW, SL, YPY, ZLC, BZ, ZWZ, XY, and HQJ performed the experiments. BW, SL, LR, ZWZ, and SSL interpreted the data and performed the statistical analysis. BW, LR, ZWZ, and SSL wrote the paper. BW, SL, LR, YPY, ZLC, BZ, ZWZ, XY, HQJ, and SSL revised the paper. All authors read and approved the final paper.

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DISCLOSURE

We declare no competing interests. All authors are aware of and agree to the content of the paper and their being listed as an author on the paper.

DATA AVAILABILITY STATEMENT

The source data of the published results can be shared; they are available from the corresponding author upon reasonable request (zengzw@mail2.sysu.edu.cn or lsstriumph@163.com).

ETHICAL APPROVAL

Approval of the research protocol by an institutional review board: The research protocols were approved by Institutional Review Board of the Sixth Affiliated Hospital of Sun Yat-sen University (Register E2021093).

Informed consent: Informed written consent was obtained from all subjects.

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