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ORIGINAL ARTICLE

High Expression of Angiopoietin-like Protein 4 in Advanced Colorectal Cancer and its Association with Regulatory T Cells and M2 Macrophages

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Abstract

Colorectal cancer (CRC) is one of the most aggressive tumours in the human digestive system. Most CRC patients have poor prognosis due to metastasis and recurrence. Angiopoietin-like 4 (ANGPTL4) is involved in tumour development. Regulatory T (Treg) cells and M2 macrophages promote tumour growth and metastasis. Herein, we explored the changes of ANGPTL4 expression in CRC patients at different stages and observed whether in situ tumour-Treg and -M2 macrophages are correlated with ANGPTL4 expression. Serum ANGPTL4 (sANGPTL4) levels of 70 CRC patients and 10 healthy controls were detected by ELISA. ANGPTL4, Foxp3 and CD163 expression levels in CRC tissues were measured by immunohistochemistry. Recombinant ANGPTL4 (rANGPTL4) proteins were further added into cell-culture systems for induction of Treg cells and M2 macrophages. The results showed both sANGPTL4 and in situ tumour-ANGPTL4 expression levels increased in Dukes C–D stage CRC patients. $Foxp3^+$ and $CD163^+$ cells in tumour tissue sections were also more intensive in Dukes C–D stage patients than in Dukes A–B stage patients. $Foxp3^+$ and $CD163^+$ cells in tumour tissues were positively correlated with both tissue and sANGPTL4 expression ($P < 0.01$). Recombinant ANGPTL4 promoted the induction of murine Treg cells and M2 macrophages ex vivo. Therefore, elevated ANGPTL4 expression could be a marker for advanced CRC. Treg cell and M2 macrophage induction could be one of the mechanisms of tumour promotion mediated by ANGPTL4.

Keywords Angiopoietin-like protein 4 · Treg · M2 macrophage · Colorectal cancer · Correlation

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Introduction

Colorectal cancer (CRC) is a malignant tumour with high morbidity and mortality; approximately 20% of patients with CRC have metastasis at the time of initial diagnosis [1, 2]. To date, the detailed mechanisms of CRC pathogenesis remain unknown. Angiopoietin-like proteins (ANGPTLs) are a family of secreted glycoproteins composed of seven members and have certain homology with angiogenins. Although ANGPTLs have a similar structure with angiogenins, they do not bind to their receptors [3, 4].

Angiopoietin-like 4 (ANGPTL4) is induced by fasting, lipid-sensing peroxisome proliferator-activated receptors, TGF-β, free fatty acids and hypoxia. Full-length ANGPTL4 protein undergoes proteolytic processing by protein convertases at the linker region, releasing the N-terminal (nANGPTL4)-coiled fragment and the monomeric C-terminal fibrinogen-like domain

(C-ANGPTL4). N-ANGPTL4 is involved in the regulation of lipid metabolism and insulin sensitivity, whereas C-ANGPTL4 plays an important role in regulating tumour growth, angiogenesis and metastasis [5–7].

ANGPTL4 has been a key regulator during cancer development through its COOH-terminal fibrinogen-like domain. C-ANGPTL4 is responsible for the increased CRC cell proliferation, survival and motility under the synergistic effects of PGE2 and hypoxia [8]. ANGPTL4 also mediates antiapoptotic effects in hepatoma cells grown in a detached state (anoikis resistance) dependent on the NADPH oxidase-mediated production of superoxide [9]. In addition, collective data show that ANGPL4 promotes vascularisation, invasion and metastasis under hypoxic conditions in tumours [7]. However, ANGPTL4 displays an anti-tumourigenic effect against tumour angiogenesis and metastasis, suggesting that the role of ANGPTL4 in cancer development is tissue dependent [7].

Regulatory T (Treg) cells and M2 macrophages are important tumour-promoting immune cells. Increased Treg cells in tumour tissues are associated with poor prognosis [10]. M2 macrophages secrete various cytokines that stimulate tumour cell proliferation and survival, including epidermal growth factor, platelet-derived growth factor and ligands of the epidermal growth factor receptor family [11, 12]. High ANGPTL4 expression in CRC patients with metastasis indicates the tumour-promoting role of ANGPTL4 in CRC [13]. Herein, we determined whether the infiltration of $Foxp3^+$ and $CD163^+$ cells was associated with ANGPTL4 expression in CRC tissues. We also analysed the direct effect of recombinant ANGPTL4 (rANGPTL4) on mouse Treg cell and M2 macrophage induction in vitro.

Materials and Methods

Patients and Biopsies

We recruited 70 CRC patients (36 males and 34 females) with age ranging from 39 years to 84 years (mean, 65.6 ± 11.6) and 10 healthy controls (7 males and 3 females, mean age 64.2 ± 9.2 years) as shown in Table 1. Before biopsy collection, patients did not undergo any chemotherapy or radiotherapy. Sera and colorectal tumour tissues were collected and stored at −80 °C. Matched para-cancer tissues

Table 1 Clinical characteristics of 70 colorectal cancer patients

Characteristics	Number	Dukes		χ2	P
		A-B	C-D		
Age				1.018	0.313
<60	16	6	10		
≥60	54	28	26		
Gender				0.061	0.806
Male	36	18	18		
Female	34	16	18		
Site				5.637	0.018*
Left	53	30	23		
Right	17	4	13		
Invasion depth				6.305	0.012*
<serosa	14	11	3		
≥serosa	56	23	33		
Lymph node metastasis				62.438	0.000**
No	36	34	2		
Yes	34	0	34		
Distant metastasis				11.019	0.001**
No	60	34	26		
Yes	10	0	10		
Differentiation				2.432	0.308
well	1	1	0		
Moderate	59	30	29		
poor	10	3	7		
CEA	13.16 ± 39.64	7.466 ± 13.01	18.50 ± 53.61	–	0.259
CA125	30.64 ± 111.7	18.76 ± 19.64	39.98 ± 148.8	–	0.462

CEA, carcinoembryonic antigen; CA125, cancer antigen 125

were around 5 cm away from the edge of cancer tissue. All tumour tissues were identified as adenocarcinoma by two pathologists. Dukes stages (A, B, C and D) of patients were classified according to cancer invasion, lymph node metastasis and distant metastasis, referring to the National Uniformity of Colon Cancer Pathology Study (1935). The study protocols were approved by the Ethics Committee of the Affiliated Hospital of Yangzhou University. All patients signed informed consents.

Reagents and Antibodies

The immunohistochemistry staining kit was from Boster Biological Technology (SA1022, Wuhan, Hubei, China) or

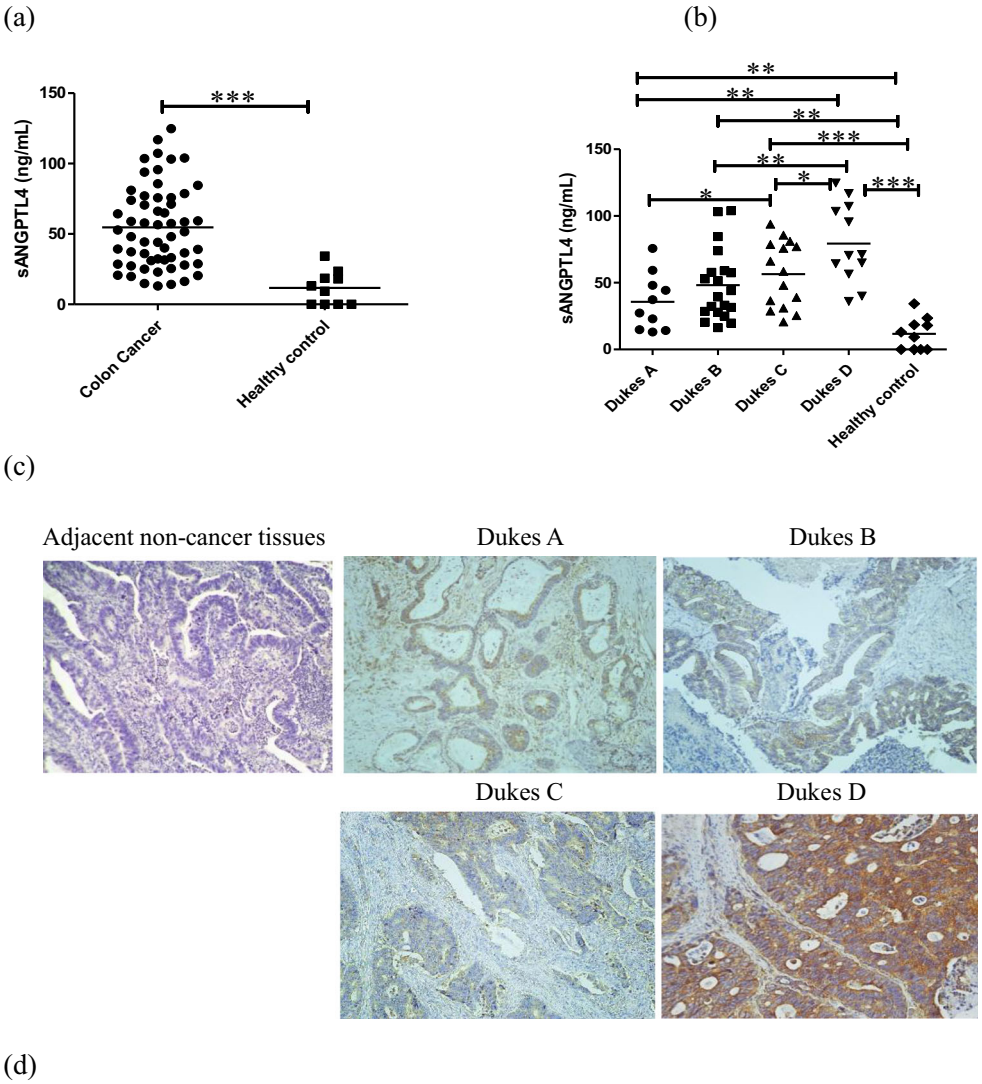


Fig. 1 Detection of ANGPTL4 levels in sera and tumour tissues of CRC patients. (**a**) sANGPTL4 levels of CRC patients and healthy controls. (**b**) ANGPTL4 levels in sera of CRC patients with different Dukes stages and healthy controls. (**c**) Representative images of ANGPTL4 staining in tumour tissues (×200). (**d**) Spearman correlation analysis of ANGPTL4 expression with colon stages. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$

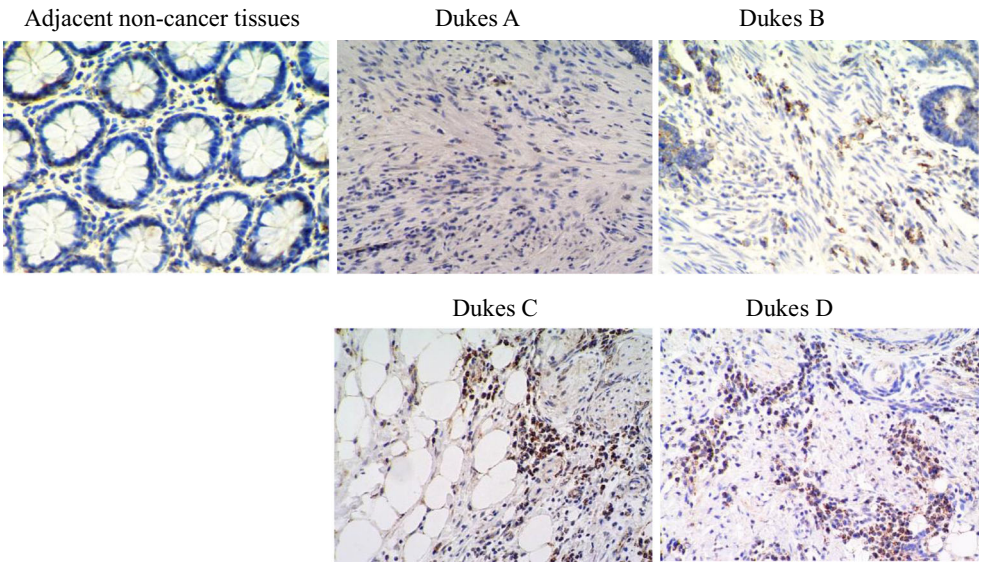
Colon Cancer stage	ANGPTL4 -	+	++	+++	Total numbers	Rs	*P*
Dukes A-B	8	11	9	6	34	0.266	⩽0.05
Dukes C-D	3	5	21	7	36		

MXB Biotechnology (KIT-9999, Fuzhou, Fujian, China). Anti-human ANGPTL4 antibody (ab196746) and anti-human Foxp3 antibody (ab191416) were from Abcam (Cambridge, UK). Anti-human CD163 antibody (bs-2527R) were from BIOSS Biological Technology (Beijing, China). Antibodies for mouse CD3 (145-2C11), CD28 (37.51), CD4 (GK1.5), CD25 (PC61), Foxp3 (MF14), F4/80 (BM8) and CD206 (C068C2) were all purchased from BioLegend (CA, USA). Recombinant mouse ANGPTL4 proteins were obtained from PeproTech (RPB019Mu01, NJ, USA). The ELISA kit for human ANGPTL4 was from Wuhan Genome Biotechnology (DRE12997, Hubei, China). Serum ANGPTL4 (sANGPTL4) level was determined according to the manufacturer's protocol.

Immunohistochemistry

All tissues were fixed overnight in 10% neutral buffered formalin, dehydrated in ethanol and embedded in paraffin. Immunohistochemical staining was performed according to standard procedures. Six fields in each section were randomly selected to be read under a microscope with high magnification. The number of positive cells in 1000 tumour cells was calculated. Section results of ANGPTL4 were scored as follows: percentage of positive cells: <5% = 0; 5%–25% = 1; 26%–75% = 2; >75% = 3. Staining intensities were assigned as follows: uncoloured or similar to the background colour = 0; light colouration, slightly higher than the background

(a)



(b)

Colon cancer stage	Foxp3$^+$ cells				Total numbers	Rs	*P*
	-	+	++	+++			
Dukes A, B	6	16	11	1	34	0.670	< 0.001
Dukes C, D	0	1	22	13	36		

Fig. 2 Detection of Treg cells in CRC tissues. (**a**) Representative staining of Foxp3 in CRC tissues at different stages (×200). (**b**) Spearman correlation analysis of Treg cells with CRC stages

colour = 1; medium colouration, significantly higher than the background colour and brownish yellow = 2; strong tan staining = 3. The combined score for each tissue sample was determined by the sum of the averaged scores: 0 to 1 being denoted as '−', 2 to 3 as '+', 4 to 5 as '++' and 6 as '+++'.

Densities of Foxp3 or CD163 cells were evaluated in stromal components of tumors. Section results of Foxp3 and CD163 were scored as follows: Ten fields magnified with 200 were randomly selected under the microscope, and $Foxp3^+$ and $CD163^+$ cell numbers in each section were counted. Positive cells were scored as follows: <5% was denoted as '−', 5% to 25% as '+', 26% to 75% as '++'and > 75% as '+++'. Two pathologists assessed the grades of staining in sections separately.

Stimulation of Treg and M2 Macrophages

C57BL/6 mice (4–6 weeks) were sacrificed, and the monocytes of their spleens were separated following the procedure from a previous study [14]. In the stimulation of Treg cells, a 24-well plate was coated with anti-CD3 antibody (10 μg/ml) in phosphate buffer saline (PBS) at 37 °C for 2 h. Then, anti-CD28 antibody (2 μg/ml), recombinant mouse TGF-β (5 ng/ml) and IL-2 (20 U/ml) were added to each well. In the stimulation of M2 macrophages, 20 ng/ml of IL-4 was added to the cell-culture systems. Splenocytes were all cultured in a 5% CO_2 incubator at 37 °C for 3 days. Different concentrations of rANGPTL4 protein (0.5 and 10 μg/ml) were simultaneously added to each cell well. The cultured cells were finally collected and labelled with the corresponding antibodies for flow cytometry. The flow-cytometric intracellular staining of Foxp3 was conducted as described previously [14].

Statistical Analysis

Continuous variables were shown as means ± standard differences. Differences between two or multiple groups

(a)

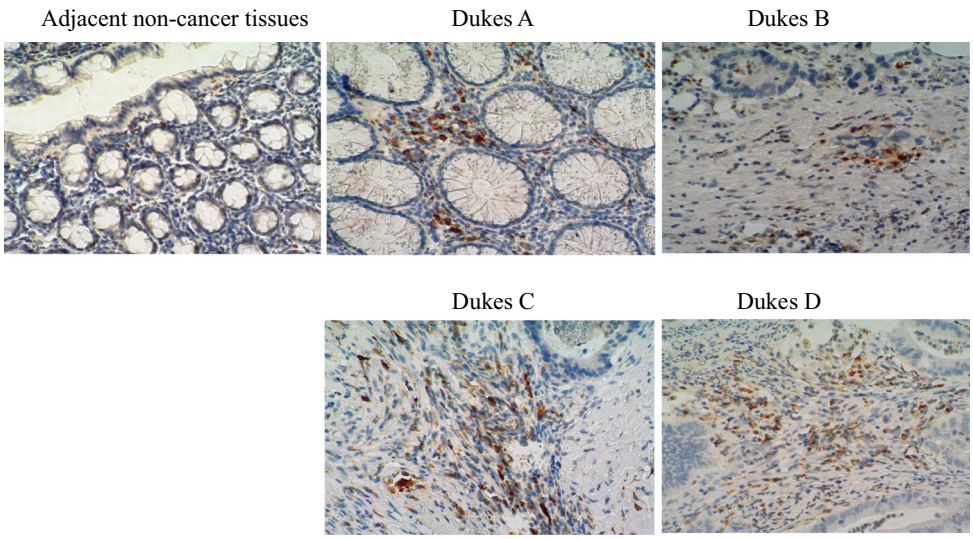
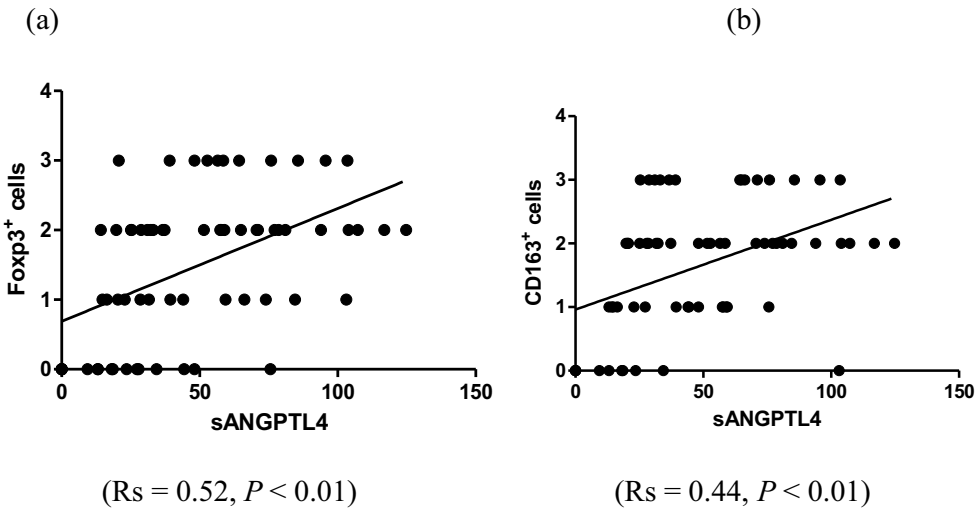


Fig. 3 Detection of M2 macrophages in CRC tissues (×200). (**a**) Representative staining of CD163 in CRC tissues at different stages. (**b**) Spearman correlation analysis of M2 macrophages with CRC stages

(b)

Colon cancer stage	$CD163^+$ cells				Total numbers	Rs	*P*
	-	+	++	+++			
Dukes A, B	1	17	15	1	34	0.696	<0.001
Dukes C, D	0	0	17	19	36		



(Rs = 0.52, $P < 0.01$) (Rs = 0.44, $P < 0.01$)

Fig. 4 Correlational analysis of ANGPTL4 expression with Treg cells and M2 macrophages. Spearman correlation analysis of sANGPTL4 expression levels with Treg cells (**a**) and M2 macrophages (**b**) in tumour tissues

were analysed using Student's *t* test or one-way ANOVA, respectively. Spearman correlation analysis was employed to compare the grade data. Significance of differences was indicated as * for $P < 0.05$, ** for $P < 0.01$, and *** for $P < 0.001$.

Results

Increased ANGPTL4 Expression in Advanced CRC Patients

Compared with those in healthy controls, the sANGPTL4 levels of CRC patients were significantly increased, as shown in Fig. 1a. The variations of sANGPTL4 in different Dukes stages were then analysed. An increasing trend of sANGPTL4 in Dukes A to D stage CRC patients was noted. The sANGPTL4 level in Dukes D stage patients was higher than that in Dukes A, B and C stage patients (Fig. 1b). We checked the ANGPTL4 expression in tumour tissues by immunohistology. Both tumour cells and their microenvironment cells were positively stained by ANGPTL4 antibody (Fig. 1c). We combined Dukes A and B stage patients into the early stage of CRC group, and Dukes C and D stage patients into the advanced stage of CRC group. The case numbers of ANGPTL4 expression levels (−, +, ++, +++) in Dukes A–B or C–D stage were calculated. Spearman correlation analysis showed that the increased ANGPTL4 expression was significantly correlated with disease progression (Fig. 1d).

Increased Infiltration of Foxp3$^+$ and CD163$^+$ Cells in Advanced CRC Patients

Treg cells promote tumour development through secreting immunoregulatory cytokines, such as IL-10 and TGF-β, or inhibiting effector responses dependent on direct contact [15]. We analysed the distributions of Treg cells in CRC tissues. The positive staining of Foxp3 generally indicated Treg cells. Compared with those in para-tumour normal tissues, more Foxp3$^+$ cells infiltrated in the interstitial tissues of CRC (Fig. 2a). The case numbers of Foxp3$^+$ infiltration grade (−, +, ++, +++) in Dukes A–B (early stage) or C–D (advanced stage) were also calculated. Spearman correlation analysis showed that the infiltration of Foxp3$^+$ cells was strongly correlated with CRC progression (Fig. 2b).

Compared with M1 macrophages, M2 macrophages promote tumour development and metastasis dependent on the secretion of IL-10, arginase, VEGF and MMP [16]. CD163 is an important biomarker of M2 macrophages. CD163$^+$ cells were clearly distributed in the interstitial tissues (Fig. 3a). More patients with high density of CD163$^+$ cells were observed (Fig. 3b), indicating that CD163$^+$ cells were associated with the development of colorectal cancer.

Correlation Study of Foxp3$^+$ and CD163$^+$ Cells with ANGPTL4

We next determined whether the infiltrations of Treg cells and M2 macrophages were correlated with sANGPTL4 levels or

Table 2 Correlational study of in situ tumor ANGPTL4 expression with Foxp3$^+$ and CD163$^+$ cells

		ANGPTL4		*P*	r^2
		−/+	++/+++		
Foxp3$^+$ cells	−/+	15	8	$P = 0.001$	0.358
	++/+++	12	35		
CD163$^+$ cells	−/+	13	5	$P = 0.001$	0.377
	++/+++	14	38		

ANGPTL4 expression of tumour tissues. Results showed that the tumour-infiltrating Treg cells were positively correlated with sANGPTL4 expression levels, as shown in Fig. 4a. M2 macrophages that infiltrated tumour tissues were also positively correlated with sANGPTL4 expression (Fig. 4b). The correlation of ANGPTL4 expression in tumour tissues with in situ Treg cells or M2 macrophages was also analysed using the Chi-square test. Statistical results showed that both $Foxp3^+$ and $CD163^+$ cells in tumour tissues were strongly correlated with high ANGPTL4 expression (Table 2), indicating that ANGPTL4 secretion associated with $Foxp3^+$ and $CD163^+$ cells in the tumour development.

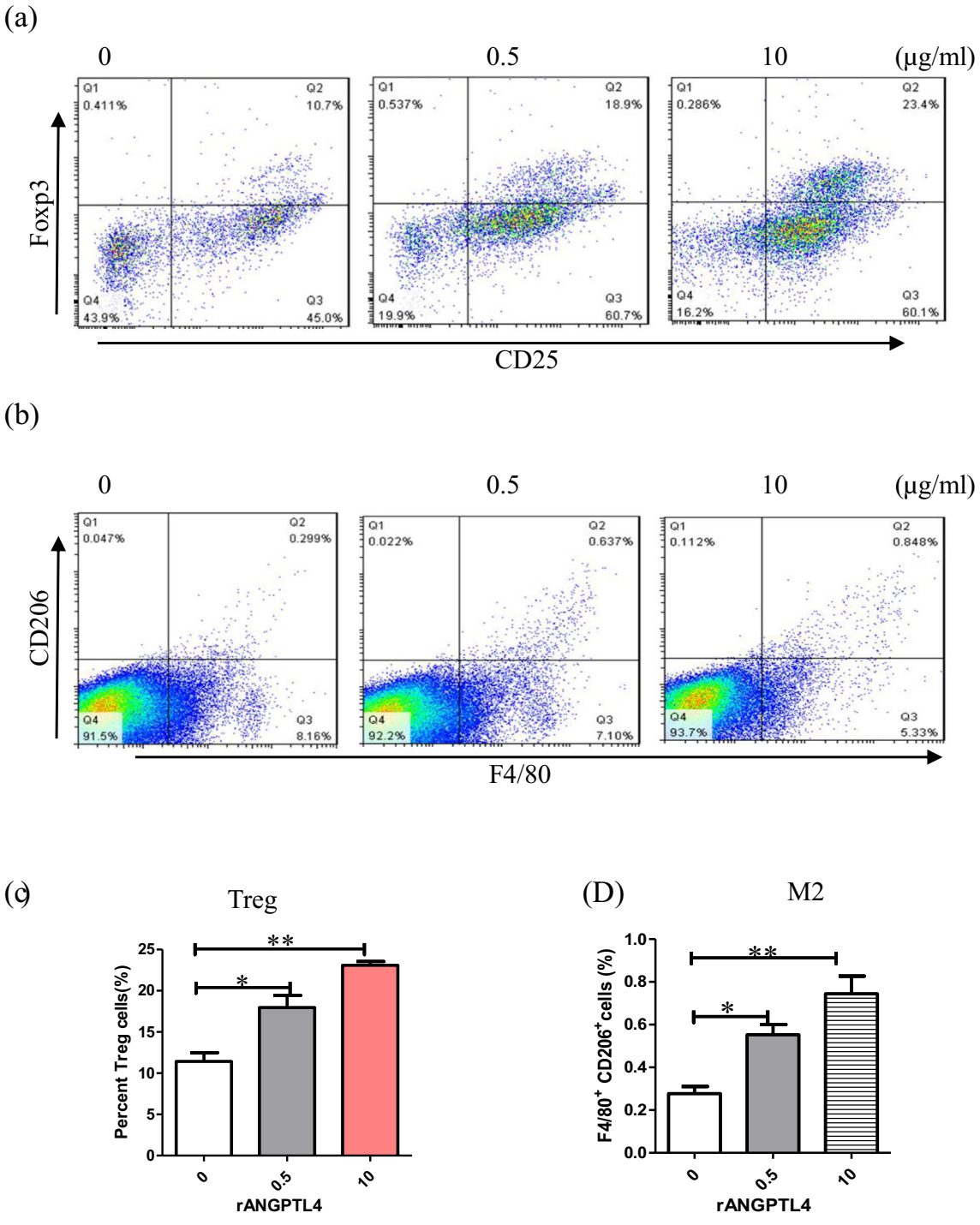


Fig. 5 Induction of murine Treg cells and M2 macrophages by rANGPTL4 ex vivo. Frequencies of $CD4^+$ $CD25^+$ $Foxp3^+$ cells (**a**) or $F4/80^+$ $CD206^+$ cells (**b**) stimulated by culture medium or rANGPTL4 for 72 h detected by flow cytometry. Statistical analysis of the variations of Treg cells (**c**) or M2 macrophages (**d**) among different groups. The experiment was performed thrice. *, $P < 0.05$; **, $P < 0.01$

Treg Cell and M2 Macrophage Induction by ANGPTL4

We observed the effects of rANGPTL4 protein on the induction of Treg cells and M2 macrophages in vitro. As expected, the addition of ANGPTL4 significantly promoted $CD25^+$ $Foxp3^+$ subset induction among $CD4^+$ cells (Fig. 5a and c). Similarly, ANGPTL4 stimulated M2 macrophages with surface markers of F4/80 and CD206 (Fig. 5b and c). The ability of ANGPTL4 to induce M2 macrophages is relatively lower than that of Treg cells. These results showed that ANGPTL4 could directly induce Treg cells and M2 macrophages.

Discussion

ANGPTL4 regulates various malignant processes, including the disruption of vascular endothelial cell–cell tight junction, promotion of tumour cell proliferation, angiogenesis, metastasis, wound healing and inhibition of tumour apoptosis [7, 17]. Herein, we confirmed the increased ANGPTL4 expression in both sera and tumour tissues in advanced CRC patients. More Treg cells and M2 macrophages were recruited into tumour tissues in the late stages of CRC. The tumour-infiltrated Treg cells and M2 macrophages were correlated with ANGPTL4 expression in sera and tumour tissues, and rANGPTL4 could promote the induction of these immune cells in vitro. Therefore, the effects of ANGPTL4 on tumour growth may also be involved with its induction of Treg cells and M2 macrophages.

Higher expression of ANGPTL4 is correlated with shorter disease-free survival rates in cancer patients [8]. Besides CRC, elevated ANGPTL4 expression has been shown in brain metastasising melanoma [18], breast cancer [19], Kaposi sarcoma [20], endometrial cancer [21], prostate cancer [22], oesophageal cancer [23], oral tongue cancer [24], hepatoma [25] and gastric cancer [26]. The roles of ANGPTL4 expression may be complex and tumour-type dependent. Some studies found that the downregulation of ANGTPL4 impairs tumour growth and metastasis, but other studies show that high ANGPTL4 levels retard tumour cell migration, invasiveness and metastasis, particularly in gastric cancer [27] and mouse tumour cells [28]. Thus, the elevated ANGPTL4 levels in sera and tumour tissues herein confirmed the tumour-promoting effect of ANGPTL4 in CRC.

Innate and acquired immune systems can combine to produce local immunosuppressive environments for tumour growth. High infiltration of Treg in the tumour microenvironment is associated with poor prognosis of multiple solid tumours [29]. The increased frequency of Treg cells is correlated with the local infiltration and extension of the tumour [30]. Treg cells directly inhibit the function of normal human CTLs, effector $CD4^+$T cells and natural killer cells [31–33]. Recruitment of Treg cells in CRC is involved with its expression of *CCR1*, *CCR8* and *TNFRSF9*, whereas cancerous epithelium expresses their ligands *CCL4* and *TNFSF9* [30]. Whether tumour-derived ANGPTL4 could directly promote the recruitment of Treg cells need more study. On the contrary, higher Treg infiltration in colorectal cancer tissues is associated a better prognosis [34, 35], particularly in CRC tissues infiltrated with low $CD8^+$T [36] or high Th17 cells [37]. Thus, high infiltration of Treg cells may have distinctive prognosis in different types of CRC.

Tumour-associated M2 macrophages promote immunosuppression and angiogenesis mainly depending on the secretion of cytokines such as arginase-I, IL-10, IL-35 and VEGF [16, 38]. M2 macrophage-derived exosomes also promote cell migration and invasion in colon cancer [39]. High $CD68^+$ macrophage density in colon cancers is negatively associated with overall survival and progression-free survival [40]. Recombinant ANGPTL4 directly promotes the induction of Treg cells and M2 macrophages in vitro, but the molecular mechanisms remain unknown. Given that ANGPTL4 may have effects on cancer metabolism via the AKT/PKB signalling [7], metabolic reprogramming of Treg cells and M2 macrophages affected by ANGPTL4 cannot be excluded.

In conclusion, despite the conflicting activities of ANGPTL4 in tumour, our study showed that increased ANGPTL4 expression levels correlated with the occurrence and progression of CRC. ANGPTL4 may mediate immune evasion by upregulating the functions of Treg and M2 macrophages. ANGPTL4 could be potentially used as a target in CRC therapy.

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Compliance with Ethical Standards

Conflict of Interest The authors have no conflicts of interest to disclose.

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