Tumour-associated macrophages mediate the invasion and metastasis of bladder cancer cells through CXCL8

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Tumour-associated macrophages (TAMs) are associated with both the progression and poor prognosis of a variety of solid tumours. This study aimed to investigate and clarify the tumour-promoting role of CXCL8 secreted by TAMs in the urothelial carcinoma microenvironment of the bladder. Immunohistochemistry (n = 55) was used to detect Chemokine (C-X-C motif) ligand 8 (CXCL8), CD163 (a TAM marker), Matrixmetalloproteinase-9 (MMP-9), vascular endothelial growth factor (VEGF), and E-cadherin in cancerous and adjacent tissues of bladder cancer patients. TAMs-like PBM (peripheral blood mononuclear)-derived macrophages were developed using in vitro experiments. T24, 5637, and UM-UC-3 were treated with conditioned medium (CM) for the experimental intervention group, without CM for the blank control group, and with CM and an anti-CXCL8 neutralizing antibody for the experimental control group, respectively. The immunohistochemical study showed that the expression of CXCL8 was significantly upregulated as the number of infiltrating TAMs increased in the tumour tissues. A high expression of CXCL8 significantly correlated with an increase in the expression of MMP-9 and VEGF and a decrease in expression of E-cadherin in the microenvironment. This revealed that TAM-derived CXCL8 is highly associated with bladder cancer migration, invasion, and angiogenesis. The concentration of CXCL8 was significantly higher in CM collected from TAM-like PBM-derived macrophages than that from THP-1 cells. In subsequent in vitro experiments, we found that CM derived from TAM-like PBM-derived macrophages can also increase the migration rate, invasiveness, and pro-angiogenic properties of tumour cells. Additionally, the effect of CXCL8 was significantly diminished by the addition of an anti-CXCL8 neutralizing antibody to CM. The infiltration of TAMs in the tumour microenvironment leads to the elevation of CXCL8, which in turn promotes the secretion of MMP-9, VEGF, and E-cadherin by bladder cancer cells. This alters the migration, invasion, and pro-angiogenic capacity of bladder cancer cells and accelerates

cancer progression.

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18 **Abstract**

19   Tumour-associated macrophages (TAMs) are associated with both the progression and poor prognosis of a

20 variety of solid tumours. This study aimed to investigate and clarify the tumour-promoting role of CXCL8 secreted

21 by TAMs in the urothelial carcinoma microenvironment of the bladder. Immunohistochemistry (n = 55) was used to

22 detect Chemokine (C-X-C motif) ligand 8 (CXCL8), CD163 (a TAM marker), Matrix metalloproteinase-9 (MMP-9),

23 vascular endothelial growth factor (VEGF), and E-cadherin in cancerous and adjacent tissues of bladder cancer

24 patients. TAMs-like PBM (peripheral blood mononuclear)-derived macrophages were developed using in vitro

25 experiments. T24, 5637, and UM-UC-3 were treated with conditioned medium (CM) for the experimental intervention

26 group, without CM for the blank control group, and with CM and an anti-CXCL8 neutralizing antibody for the

27 experimental control group, respectively. The immunohistochemical study showed that the expression of CXCL8

28 was significantly upregulated as the number of infiltrating TAMs increased in the tumour tissues. A high expression

29 of CXCL8 significantly correlated with an increase in the expression of MMP-9 and VEGF and a decrease in

30 expression of E-cadherin in the microenvironment. This revealed that TAM-derived CXCL8 is highly associated with

31 bladder cancer migration, invasion, and angiogenesis. The concentration of CXCL8 was significantly higher in CM

32 collected from TAM-like PBM-derived macrophages than that from THP-1 cells. In subsequent in vitro experiments,

33 we found that CM derived from TAM-like PBM-derived macrophages can also increase the migration rate,

34 invasiveness, and pro-angiogenic properties of tumour cells. Additionally, the effect of CXCL8 was significantly

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36 microenvironment leads to the elevation of CXCL8, which in turn promotes the secretion of MMP-9, VEGF, and E-

37 cadherin by bladder cancer cells. This alters the migration, invasion, and pro-angiogenic capacity of bladder cancer

38   cells and accelerates cancer progression.

39   **1. INTRODUCTION**

40   Tumorigenesis is a highly complex process involving multiple factors and specific subsets of macrophages have

41   been found to be indeed involved in tumorigenesis. (Gordon et al. 2017). In the tumour microenvironment,

42   macrophages with the M2 phenotype polarized by IL-4, IL-10, etc., are often referred to as "tumour-associated

43   macrophages" (TAMs). TAMs are a major component in leukocyte infiltration in tumour tissues (Hao et al. 2012;

44   Noy & Pollard 2014). CD163 (a hemoglobin scavenger receptor) is a unique TAM marker (Almatroodi et al. 2016;

45   Barros et al. 2013). After recruiting tumour-derived chemokines (mainly Chemokine (C-C motif) ligand 2 (CCL2)

46   and colony-stimulating factor 1 (CSF-1) (Noy & Pollard 2014)), TAMs are often confined to the perivascular region

47   or the tumour-infiltrating margin. Upon arrival, they can secrete pro-migratory factors (e.g. epidermal growth factor,

48   or EGF), degrade extracellular matrix proteolytic enzymes, accelerate tumour migration, and induce tumour invasion

49   and metastasis (Huang & Feng 2013; Pollard 2004; Quail & Joyce 2013). It has been shown that in solid tumours,

50   including bladder cancer, an increased number of CD163^{+} TAM infiltrates is positively correlated with a poor clinical

51   outcome (Komohara et al. 2014; Lewis & Pollard 2006; Wang et al. 2015).

52   Solid tumours and their surrounding areas are composed of inflammatory cells as well as various types of

53   chemokines and cytokines (Stadler et al. 2015). CXCL8 is considered a prototypic chemokine belonging to the CXC

54   family, which is responsible for the recruitment and activation of neutrophils and granulocytes to inflammation sites

55   (Waugh & Wilson 2008). CXCL8's role in cancer is widely recognized. It not only participates in tumour

56   angiogenesis, but also activates MMP to promote metastasis-related tissue remodeling (Azenshtein et al. 2005; Kim

57   et al. 2001). The expression of CXCL8 in urine specimens and the tumour microenvironment of bladder cancer patients

58   is significantly higher than that of healthy volunteers (Pignot et al. 2009; Sheryka et al. 2003), and the overexpression

59   of CXCL8 is positively correlated with significantly reduced survival of patients overall (Zhang et al. 2014). It has

60   been shown that TAMs in the solid tumour microenvironment can secrete CXCL8 in large amounts, directly

61   contributing to the progression of pancreatic ductal carcinoma, papillary thyroid cancer, colorectal cell carcinoma,

62   and esophageal squamous cell carcinoma (Chen et al. 2018; Fang et al. 2014; Hosono et al. 2017; Lee et al. 2012).

63   However, little is known about the role of CXCL8 derived from TAMs in the para-epithelial carcinoma network of

64   the human urinary tract in the bladder tumour microenvironment. In this study, we focused on CXCL8 secreted by

65   TAM-like PBM-derived macrophages, and determined the critical role and biological effects of TAM-derived CXCL8

66   on bladder cancer cell production.

67   **2. MATERIALS AND METHODS**

68   2.1 Patient selection and data collection

69   The Second Hospital of Lanzhou University approved this research, and all participants provided informed consent

70   for this study. Immunohistochemical staining was performed on 55 pathologically diagnosed bladder cancer patients

71   who underwent total or partial cystectomy between September 2017 and December 2018. All hematoxylin and eosin

72   (H&E)-stained specimens obtained by initial cystectomy were reassessed independently by experienced

73   uropathologists.

74   2.2 Immunohistochemical staining and quantification

75   Paraffin-embedded sections for immunohistochemical detection were first deparaffinized in xylene and rehydrated

76   in ethanol, and added to a pressure cooker with 0.01 M citrate (pH = 6.0) buffer and antigen recovery. After pressure

77   was reached, the sections were incubated in the pressure cooker for 2 min. Endogenous peroxidase activity was

78   blocked using 1% hydrogen peroxide in methanol according to the Streptavidin-Peroxidase Detection Kit (ZSGB-

79   BIO, China) after blocking in 1% bovine serum albumin (BSA) for 10 min. Slides were incubated overnight at 4 ° C

80   with anti-human CXCL8 antibody (Abcam, CA, goat polyclonal, diluted 1/1000 in 1% BSA), anti-CD163 antibody,

81   anti-VEGF antibody, anti-MMP-9 antibody, and anti-E-cadherin antibody (Santa Cruz, CA, goat polyclonal, diluted

82   1/250 in 1% BSA), respectively. Slides were then incubated with biotinylated anti-goat IgG secondary antibody for

83   10 min at room temperature. Sections were subsequently stained with 3, 3`-diaminobenzidine (DAB; Vector

84   Laboratories), counterstained with hematoxylin (Solarbio, Peking, China), dehydrated, and coverslipped. The results

85   were observed under a microscope (OLYMPUS BH-2 Microscopes, USA), and the positive staining rate was counted.

86   The semi-quantitative methods used for immunohistochemistry results were as follows: CD163-positive round cells

87   were counted in cancerous areas using at least three independent high-power microscopic fields (HPF; 400×, 0.0625

88   μm^2). The mean number of CD163-positive cells was considered the number of tumour-infiltrating TAMs. To quantify

89   the expression levels of CXCL8, MMP9, VEGF, and E-cadherin in bladder cancer cells, tumour cells were counted

90   in at least three independent fields (HPF; 400×, 0.0625 μm^2), and that number was divided by the total cancer cells to

91   calculate the percentage of positive cells (1–100%). The evaluation was carried out blindly by two investigators,

92   without knowledge of the patients' outcome or other clinic pathological characteristics.

93   2.3 Cell lines and reagents

94   Three bladder cancer cell lines, 5637, UM-UC-3, and T24, as well as acute monocytic leukemia cell line THP-1

95   and EA·hy926 (Cell Bank of the Chinese Academy of Sciences, Peking, China) were used in the present study. Cell

96   lines were maintained in RPMI-1640 medium (Gibco, Thermo Fisher Scientific, USA) supplemented with 10% fetal

97 bovine serum (Pan Biotech, Germany), 100 units/mL penicillin, and 100μg/mL streptomycin in a standard humidified

98 incubator at 37°C in 5% CO_2. Phorbol-12-myristate-13-acetate (PMA) was purchased from Multis Sciences

99 (LIANKE) Biotech (Hangzhou, China). Recombinant protein IL-4 (PeproTech, USA) was also used in the described

100 experiments.

101 2.4 Generation of TAMs from THP-1

102 To generate TAMs from monocytic THP-1, cells were seeded 1×10^6 cells in a dish and treated with PMA (200nM)

103 for 24 h for differentiation into resting macrophages (M0 cells). For M2 polarization, cells were treated with IL-4 (20

104 ng/mL) for an additional 48 h (Genin et al. 2015; Mantovani et al. 2004). qRT-PCR analysis for CD163 was performed

105 to confirm TAM generation.

106 2.5 Enzyme-linked immunosorbent assay (ELISA)

107 A human CXCL8 kit and MMP-9 kit were purchased from Neobioscience Technology Company, China, and

108 ELISA was performed according to the manufacturer's instructions. Both standard and tissue samples were added to

109 a 96-well plate with 0.1 mL of biotinylated antibody and incubated at 37 ° C for 60 minutes. After gentle washing,

110 enzyme conjugate working solution was added to each well and the samples incubated for 30 min. Chromogenic

111 substrate (TMB) was then added and the samples incubated for 15 min in the dark. After stopping with stop solution,

112 the optical density at 450 nm was quantified using a microplate reader (Bio Tek Instruments, USA) for further analysis.

113 2.6 Quantitative real-time polymerase chain reaction (qRT-PCR)

114 RNA was extracted from cells using TRIzol (Takara, Japan) as per the manufacturer's instructions. Conversion to

115 cDNA was achieved using cDNA PrimeScript™ RT Master Mix (Perfect Real Time) (Takara, Japan) in a reverse

116 transcription PCR instrument (Bio-Rad Laboratories, USA). Quantitative qRT–PCR was carried out using the CFX

117   Real-Time PCR System (Bio-Rad Laboratories, USA) in a 15-μl reaction volume containing first-strand cDNA, TB

118   Green™ Premix Ex Taq™ II (Tli RNaseH Pluse). Primer sets can be found in Table 1. Relative fold changes in mRNA

119   levels were calculated after normalization to GADPH using the comparative Ct method.

120   2.7 Western blot analysis

121   After the treated cells were collected and washed with PBS and RIPA buffer lysis, they were placed on ice for 30

122   minutes. The concentration of supernatant protein was determined using the BCA method (Solarbio, Peking, China)

123   after centrifugation at a speed of 14,000 r.p.m. for 15 minutes. The same amount of total protein was resolved using

124   SDS-PAGE and transferred to the PVDF membrane. Antibodies to the target protein were tested overnight at 4°C.

125   The primary antibodies used in this study were anti-E-cadherin (dilution,1:100), anti-VEGF (dilution,1:200), and anti-

126   actin antibody (dilution, 1:1000; ZSGB-BIO, China) as an internal loading control. After incubation with IRDye®

127   near-infrared fluorescence secondary antibody, the protein was detected using Odyssey Near-Infrared Fluorescence

128   Imaging Systems (LI-COR Biosciences, USA).

129   2.8 Cell invasion assay using conditioned media obtained from TAMs

130   THP-1-derived TAMs were seeded in serum-containing media onto cell culture dishes at a density of 1,000,000

131   cells/dish. Twenty-four hours later, the medium was replaced with serum-free RPMI1640 medium, and cells were

132   incubated for an additional 48h. The CM was collected from manipulated TAMs, followed by centrifugation to remove

133   cells or cell debris. The invasion assay was performed using Costar® Transwell® inserts (Corning Incorporated, USA)

134   according to the manufacturer's directions, and 24-well plates with 8-μm pore membranes were lightly coated with

135   2mg/mL Matrigel (Solarbio, Peking, China). T24, 5637, and UM-UC-3 cells were added to each insert at a density of

136   30,000 cells/well in the CM described above. The lower chamber contained RPMI1640 medium with 10% FBS as a

137   chemoattractant. The cells were maintained in a humidified incubator with 5% CO_2 at 37°C for 48 h. Invading cells

138   on the membrane were then fixed using paraformaldehyde, stained with 0.1% crystal violet (Solarbio, Peking, China),

139   and positively stained cells were counted. The cell numbers counted from five random fields were averaged.

140   2.9 Wound healing assay

141   Cells were seeded in six-well plates and incubated overnight. A vertical bar was drawn using a mark pen on the

142   back of the six-well plate, and a 1mL pipette tip was used to transversely scratch the cell monolayer. After washing

143   the scraped cells from the wells with PBS, serum-free medium, CM, and CM with added anti-CXCL8 neutralizing

144   antibody were added to the wells. Tumour cell migration was observed at 0, 6, 12 and 24 h. Wound healing percentage

145   was calculated using Image J software.

146   2.10 Vascular endothelial cell tube formation experiment

147   Matrigel (8.7mg/mL) was placed in a refrigerator at 4°C to slowly melt overnight, and 50μL was spread in each

148   well of a 96-well plate and placed in an incubator at 37 °C for 1 hour. The EA·hy926 cells were prepared into a cell

149   suspension of 2×10^5 cells/mL, which was mixed well and added to a 96-well plate at a volume of 100μL per well. The

150   CM prepared in advance was then added at a volume of 50μL per well, incubated in an incubator at 37 °C and 5%

151   CO_2, and observed after 8 hours (Arnaoutova & Kleinman 2010; Lee & Kang 2018; Ma et al. 2017). We observed the

152   tube formation of endothelial cells and counted the number of vessels and visible nodes.

153   2.11 Statistical Analysis

154   All experimental results were expressed as mean ± SD and analyzed for statistical significance by a two-tailed

155   Student's t-test. The interrelationship was examined using Spearman's analysis. Data were statistically analyzed and

156   plotted using IBM SPSS version 23 (SPSS Inc., Chicago, IL, USA) and Prism software version 5 (San Diego, CA,

157   USA), respectively. A *p*-value < 0.05 was considered statistically significant.

158   **3. RESULTS**

159   3.1 The association between the expression of CXCL8 in bladder cancer and clinicopathological features

160   The clinicopathological characteristics of 55 bladder cancer cases are outlined in Table 2. Representative pictures

161   of immunohistochemical staining for CXCL8, CD163, MMP9, VEGF, and E-cadherin are shown in Figure 1A-

162   J. Since CXCL8 is mainly expressed in the cytoplasm of tumour cells, the expression levels were significantly higher

163   in bladder cancer tissues than in normal tissues (Figure 1K). Strong CXCL8 expression was observed in the tumor

164   tissue of high-grade tumors, while low-grade tumors had relatively lower expression levels. The expression of CD163

165   (a marker of TAMs), MMP9, VEGF, and E-cadherin was quantitatively assessed and compared with the CXCL8

166   expression score. The results showed that a high expression of CXCL8 in tumour tissues was associated with a high

167   infiltration of TAMs, high expression of VEGF and MMP9, and a low expression of E-cadherin (Figure 1L-O). When

168   compared to clinicopathological variables, a higher CXCL8 expression score was associated with higher T category

169   and tumor grade (Table 2). These findings suggest that the infiltration of TAMs and the expression of CXCL8 in

170   bladder cancer exhibit the aggressiveness of the remodeled stroma.

171   3.2 TAM-like PBM-derived macrophages can secrete CXCL8

172   To demonstrate how macrophages in the microenvironment can secrete CXCL8, we used TAM-like PBM-derived

173   macrophages with IL-4-induced M2 phenotype (Figure 2A-C). The TAM-like PBM-derived macrophages were

174   cultured in a serum-free medium, which was collected daily. The amount of CXCL8 secreted in the collected culture

175   medium was then measured using an ELISA experimental method. It can be concluded that different concentrations

176   of IL-4 in vitro induce different amounts of CXCL8 secreted by TAM-like PBM-derived macrophages (Figure 2D).

177   The highest amount of CXCL8 was secreted when the concentration of IL-4 was 20 ng/mL (Figure 2E). After

178   analyzing the expression of CXCL8 and CD163 using qRT-PCR, we concluded that CXCL8 expression significantly

179   increased (Figure 2F) and TAM-like PBM-derived macrophages induced by IL-4 significantly expressed the specific

180   M2 macrophage marker CD163 (Figure 2G).

181   3.3 CXCL8 derived from TAM-like PBM-derived macrophages promotes the invasion of bladder cancer

182   To examine the potential consequences of recruiting macrophages into bladder cancer tumour microenvironments,

183   we investigated the potential effect of TAM-derived CXCL8 on bladder cancer cell invasion. We tested invasion

184   ability after culturing bladder cancer cells 5637, T24, and UM-UC-3 with/without CM, using 8μm membrane inserts

185   as described in the Materials and Methods. The results show that CM increases bladder cancer cells' invasion capacity,

186   and this effect can be suppressed using an anti-CXCL8 neutralizing antibody (Figure 3A-J). RNA subsequently

187   extracted from bladder cancer cells after the CM treatment described above showed that the expression of MMP-9

188   had significantly increased in the treated cells (Figure 3K). This was also demonstrated by ELISA at the protein level

189   (Figure 3L), indicating that CXCL8 can promote bladder cancer invasion by stimulating bladder cancer cells to secrete

190   MMP-9.

191   3.4 CXCL8 derived from TAM-like PBM-derived macrophages increases the migration of bladder cancer cells

192   We conducted a wound healing experiment to investigate the effect of CXCL8 on bladder cancer cell migration.

193   We planted 5637, T24, and UM-UC-3 bladder cancer cells on a six-well plate, attached them to the wall for 24 hours,

194   and then added CM (Figure 4A-R). We found that the cell migration increased significantly after the addition of CM.

195   After neutralizing CXCL8 in CM with anti-CXCL8 neutralizing antibody, the accelerated migration rate was observed

196   to be significantly reduced. This suggests that CXCL8 can promote the migration of cancer cells (Figure 4S).

197   3.5 CXCL8 derived from TAM-like PBM-derived macrophages promotes the formation of blood vessels in tumours

198   Angiogenesis is associated with the benign-malignant transition of tumours, with resulting blood vessels providing

199   tumour cell nutrients and oxygen for their propagation, invasion, and metastasis. The blood vessel formation process

200   is complex, and TAMs are one of the major contributors (Lin & Pollard 2007). To investigate the proangiogenic ability

201   of CXCL8 derived from TAM-like PBM-derived macrophages, we used the serum-free RPMI-1640 and CM

202   described above to culture T24 cells for 24 h, and extracted the culture medium, which was centrifuged to become

203   new CM (called CM-C and CM-T24, respectively). Using the endothelial cell tube formation assay described above,

204   we added CM-C (as control) and CM-T24 as EA·hy926 cells were added to the 96-well plate. After 8 h of culture, we

205   observed significantly more endothelial cell tube formation in wells with CM-T24 added than in those with CM-C.

206   We used CM to culture T24 cells with an additional anti-CXCL8 neutralizing antibody, and then cultured EA·hy926

207   cells in the extraction medium (CM-T24-NA) described above. We found that the tube formation of EA·hy926 cells

208   was significantly inhibited (Figure 5A-D). Total RNA and total protein were then extracted from the CM-treated

209   bladder cancer cells. QRT-PCR and WB analysis showed that the expression level of VEGF in CM-treated bladder

210   cancer cells increased, indicating that CM can induce angiogenesis by promoting the secretion of VEGF in bladder

211   cancer cells. The inhibition of CXCL8 with the CXCL8-neutralizing antibody reduced the ability of CXCL8 to

212   promote VEGF secretion in bladder cancer cells, suggesting that CXCL8 in CM plays a role in this process (figure

213   5E-G).

214   **4. DISCUSSION**

215   It has been well-established that the microenvironment of solid tumors is constituted of malignant cells and

216   some non-malignant mesenchymal cells (Balkwill & Mantovani 2001; Nilendu et al. 2018). Among these non-

217   malignant cells, macrophages play an important role in promoting tumour migration, invasion, and new blood

218   vessel formation. TAMs in the tumour microenvironment can lead to pro-tumorigenic inflammation, which plays

219   an important role in tumorigenesis (Mantovani & Sica 2010), but TAMs can also express both chemokines and

220   cytokines to promote an immunosuppressive tumour microenvironment (Guo et al. 2016). The infiltration of

221   TAMs in primary tumours is associated with a poorer prognosis across almost all tumours (Mantovani et al.

222   2017), and when macrophage infiltration in the tumour microenvironment is blocked using a colony stimulating

223   factor-1 (CSF-1) or colony stimulating factor-1 receptor (CSF-1R) inhibitor, the tumour burden in patients can

224   be significantly reduced (Ries et al. 2014). Although it has been established that TAMs are associated with the

225   progression of solid tumours and poor patient prognoses, the specific TAM mechanism in tumour cells has not

226   been fully explained. This study found that the infiltration of TAMs in bladder cancer patients significantly increased

227   when compared with the normal control, and TAM levels positively correlated with the expression of CXCL8 in

228   bladder cancer tissues. This demonstrates that a high expression of CXCL8 is strongly associated with the infiltration

229   of TAMs, and studies have shown similar findings in other cancers (Fang et al. 2014; Lin et al. 2019).

230   The CXCL8/CXCL8R axis plays an important role in human cancer and can contribute to tumour progression

231   in multiple ways (Ha et al. 2017; Liu et al. 2016). Under the influence of various stimuli in the tumour

232   microenvironment, stromal cells produce CXCL8, which may affect the invasiveness and metastatic potential of

233   cancer cells (Mukaida 2003). Blood vessels in tumours provide tumour cells with the necessary nutrients and

234   oxygen, creating convenient conditions for tumour cell growth and invasion (Cao 2005). When stimulated with

235 CXCL8, vascular endothelial cells begin an angiopoiesis process characterized by the secretion of MMP which

236 breaks down the extracellular matrix (Li et al. 2003). CXCL8 can significantly increase tumour vessel density

237 in prostate cancer, which has a very strong angiopoiesis effect (Araki et al. 2007; Inoue et al. 2000a; Masuya et

238 al. 2001). In the present study, we observed that the high expression of CXCL8 in bladder cancer tissues

239 positively correlated with the elevated expression of VEGF, suggesting that CM derived in vitro from TAM-like

240 PBM can promote the secretion of VEGF from bladder cancer cells and blood vessel formation. Therefore, the

241 synergistic effect of CXCL8 and VEGF can promote tumour angiogenesis (Masuya et al. 2001).

242 CXCL8-induced MMP-9 promotes extracellular matrix degradation (Inoue et al. 2000b), which not only

243 provides the necessary conditions for angiogenesis, but also plays an important role in cancer cell invasion and

244 metastasis. In the present study, a high expression of MMP-9 in cancer tissues was observed by

245 immunohistochemistry, and there was a positive correlation with the increase of CXCL8 expression. Moreover,

246 CM derived from TAM-like PBM-derived CXCL8 could contribute to MMP-9 expression in bladder cancer

247 cells, suggesting that it is also involved in bladder cancer invasion and metastasis. Additionally, when the effect

248 of CXCL8 was inhibited using an anti-CXCL8 neutralizing antibody, we observed that the ability of bladder

249 cancer cells to promote angiogenesis, invasion, and metastasis was significantly reduced.

250 Epithelial-mesenchymal transition (EMT), a process of transition from epithelial cell phenotype to active

251 mesenchymal cells, plays a key role in tumour progression. TAMs help coordinate this process, which includes

252 the loss of cell-cell contact and the acquisition of a migratory phenotype. The regulation of EMT involves many

253 cytokines and chemokines, including CXCL8 (Cheng et al. 2014). IHC assays on clinical specimens in the

254 present study concluded that the expression of E-cadherin decreased under the influence of CXCL8. Moreover,

255 after extracting RNA and protein from bladder cancer cells treated with and without CM, we found that the

256 expression of E-cadherin significantly decreased in bladder cancer cells treated with CM, an effect that could be

257 resisted using an anti-CXCL8 neutralizing antibody (Among bladder cancer cells, T24 has the highest degree of

258 malignancy, which may lead to low or even no expression of E-cadherin in T24). This demonstrates that CXCL8

259 derived from TAMs can increase bladder cancer cell migration by decreasing the expression of E-cadherin

260 (Figure 4T-V).

261 This study found that infiltrating TAMs can modify the bladder tumor microenvironment by promoting tumor

262 progression with CXCL8. A contrasting study has shown that CXCL8 secreted by tumor cells activates

263 proangiogenic and anti-apoptotic pathways by promoting the expression of VEGFA and bcl-2 at the invasion

264 front, which confirms the key role of CXCL8 in tumor progression (Kumar et al. 2018). A similar autocrine

265 mechanism of this CXC chemokine has also been reported (Bandapalli et al. 2011; Matsuo et al. 2009). By

266 observing immunohistochemical section staining, we found that bladder cancer cells could also secrete CXCL8,

267 meaning that there is complex intercellular communication in the tumor microenvironment involving autocrine

268 and paracrine chemokines. Due to the complexity of the tumor microenvironment and its components, tumor

269 invasion, migration, and angiogenesis may be determined by more than one factor or pathway. Therefore, a

270 macroscopic view of the tumor microenvironment would be effective. This study focused on the role of CXCL8

271 secreted by TAMs in promoting bladder cancer, but further work is needed to determine the full effect of

272 microenvironment on tumors.

273 **CONCLUSION**

274   In summary, TAM-derived CXCL8 can promote the expression of MMP-9, VEGF, and E-cadherin in bladder

275   cancer cells, causing changes in bladder cancer cell migration, invasion, and pro-angiogenic ability, and leading

276   to the progression of bladder cancer. The inhibition of CXCL8-signaling derived from TAMs may be a promising

277   therapeutic approach in the treatment of bladder cancer.

278   **Acknowledgements**

279   **None**

280   **Reference**

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419

qRT-PCR primers (human)

1 **Tables 1**

2 qRT-PCR primers (human)

CXCL8	Forward	CAAGCTGGCCGTGGCTCT
	Reverse	TGGGGTGGAAAGGTTTGGAGT
CD163	Forward	CCGGGAGATGAATTCTTGCCT
	Reverse	GGCCTCCTTTTCCATTCCAGAAA
MMP-9	Forward	GTACTCGACCTGTACCAGCG
	Reverse	TTCAGGGCGAGGACCATAGA
VEGF	Forward	GAGTACCCTGATGAGATCGAGT
	Reverse	ATTTGTTGTGCTGTAGGAAGCT
E-cadherin	Forward	ATGGCTGAAGGTGACAGAGC
	Reverse	CACCTTCCATGACAGACCCC
GADPH	Forward	CAGGA GGCAT TGCTG ATGAT
	Reverse	GAAGG CTGGG GCTCA TTT

Clinicopathologic characteristics of 55 cases with bladder cancer

Table 2.

Clinicopathologic characteristics of 55 cases with bladder cancer

Variables	Cases	CXCL8 expression in tumor Number of cases (percentage)		P
		Low	High	
Total	55	18	37	
Age				0.78
<65	29	9 (30%)	20 (70%)	
≥65	26	9 (35%)	17 (65%)	
Sex				0.31
Male	44	13 (30%)	31 (70%)	
Female	11	5 (45%)	6 (55%)	
Tumour size				0.41
less than 3 cm	9	4 (44%)	5 (56%)	
3 cm and more	46	14 (30%)	32 (70%)	
Tumour stage				0.01*
<T2	13	9 (69%)	4 (31%)	
≥T2	42	9 (21%)	33 (79%)	
TumoUr grade				0.02*
Low grade	6	4 (65%)	2 (35%)	
High grade	49	11 (22%)	36 (78%)	
Lymph node metastasis				0.13
Negative	23	10 (44%)	13 (56%)	
Positive	33	8 (25%)	25 (75%)	
Vascular invasion				0.73
No	38	13 (42%)	25 (66%)	
Yes	17	5 (29%)	12 (71%)	
Distant metastasis				0.93
No	31	10 (33%)	21 (67%)	
Yes	24	8 (31%)	16 (69%)	

1   P value was analyzed by a chi-square test; * indicates $P < 0.05$ with statistical significance.

Figure 1

Recruitment of tumor-associatedmacrophages and CXCL8 expression in human bladder cancer, and the correlation between the expression of CXCL8 and the expression of MMP-9, VEGF, and E-cadherin.

(A-J) Representative expression status of CXCL8, CD163, MMP-9, VEGF and E-cadherin in human bladder cancer and para-cancertissues. All images were captured at 100× and 400× magnification. (K) Expression of CXCL8 in bladder cancer and para-cancertissues. In the box-and-whisker plot, significance was assessed by the paired student's t test. **** $p <$ 0.0001. (L-O) The interrelationship between the percentage of CXCL8 positive cancer cells and (L) the number of infiltrated TAM, (M) percentage of MMP-9 positive cells, (N) percentage of VEGF positive cells, and (O) percentage of E-cadherin positive cells using Spearman's correlation. Spearman r was found to be 0.41, 0.47, 0.57, -0.42, respectively. (95% confidence interval, 0.16-0.61, 0.24-0.66, 0.36-0.73, -0.61- -0.17, respectively).
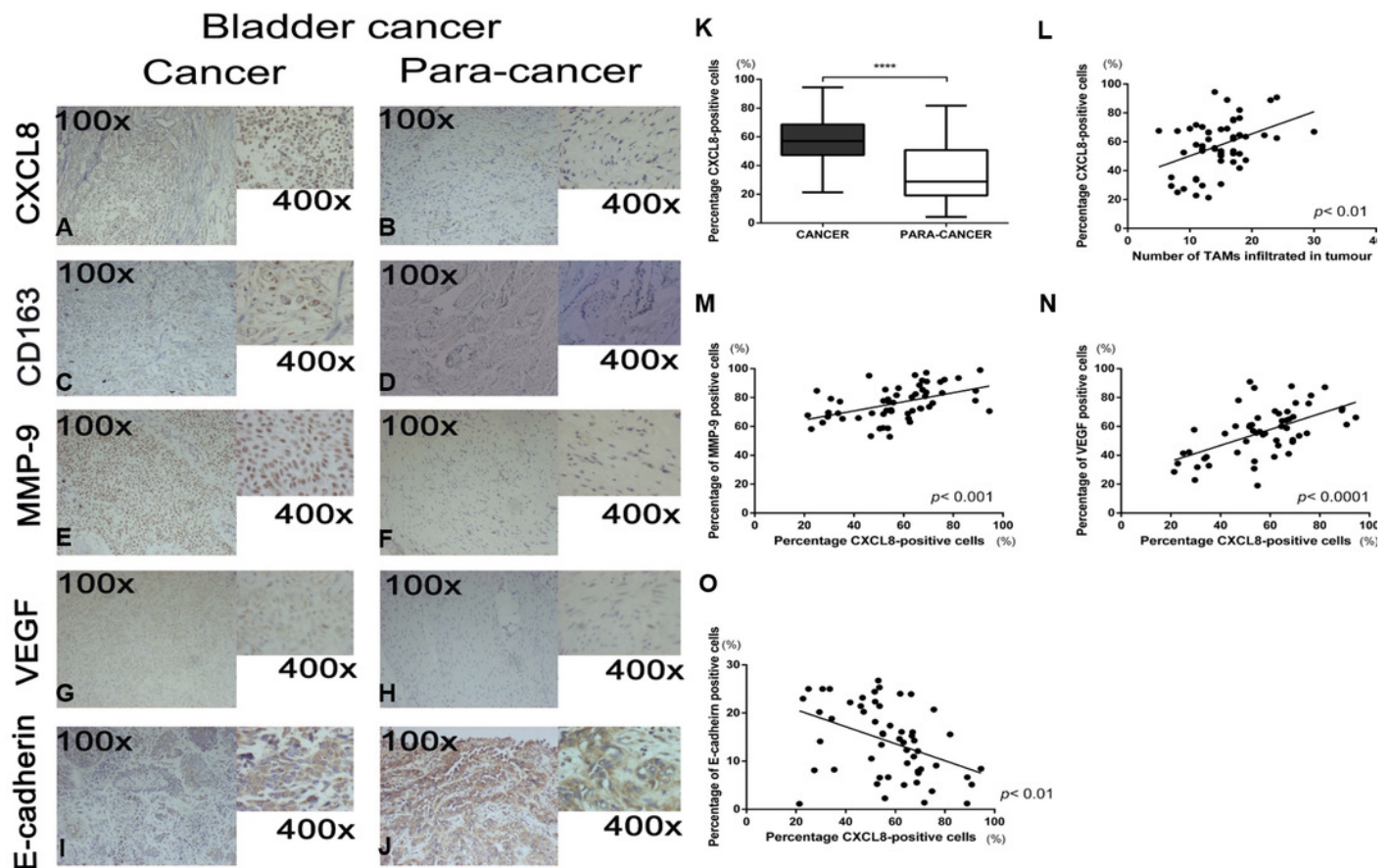
Figure 2

IL-4-induced TAM-like PBM-derived macrophages can secrete CXCL8.

(A-C) Morphological changes of THP-1. PMA (200nM, 24h) and IL-4 (20ng/mL, 48h) sequentially induce the formation of TAMs-like PBM-derived macrophages. Prior to treatment, THP-1 cells were round, floating, and did not attach to the bottom surface of the flask; after treatment with PMA and IL-4, they adhered to the bottom surface with abundant cytoplasmic processes. (D) After M0 induction using different concentrations of IL-4, the concentration of CXCL8 in serum-free medium was measured over time by ELISA. ** $p < 0.01$, *** $p < 0.001$. (E) When the concentration of IL-4 was 20 ng/mL, the amount of CXCL8 secreted by TAMs-like PBM-derived macrophages was the greatest. (F) CXCL8 mRNA expression level was determined by qRT-PCR. (G) The expression level of CD163 in TAM-like PBM-derived macrophages. Expression levels are expressed as the mean ± SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.
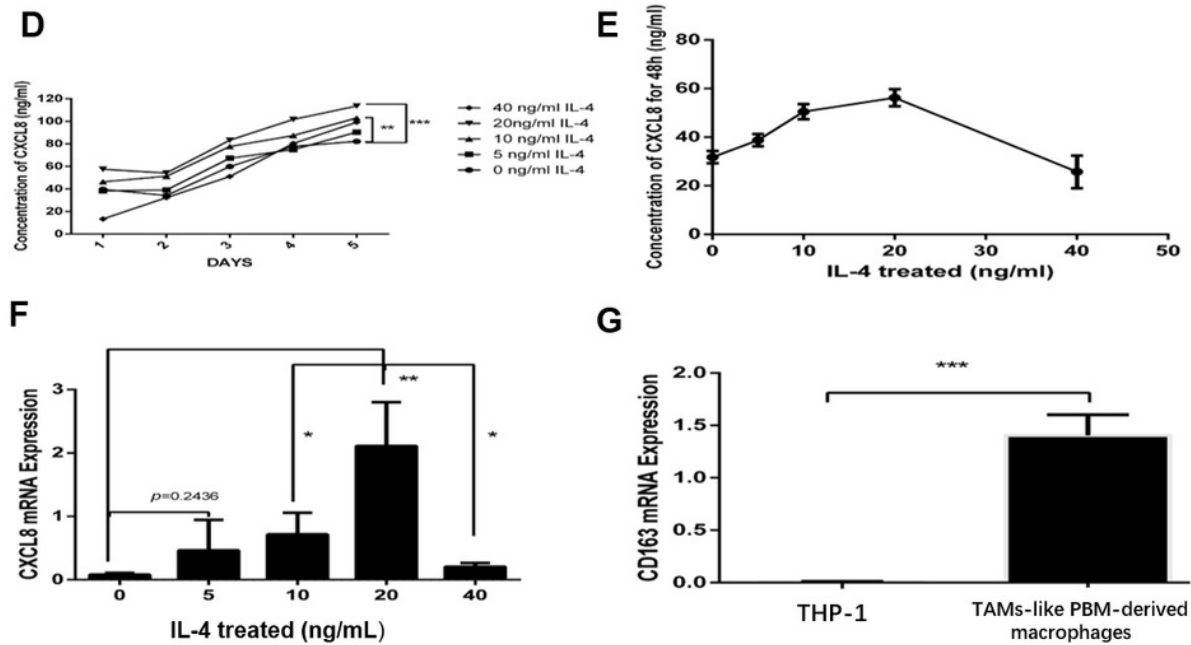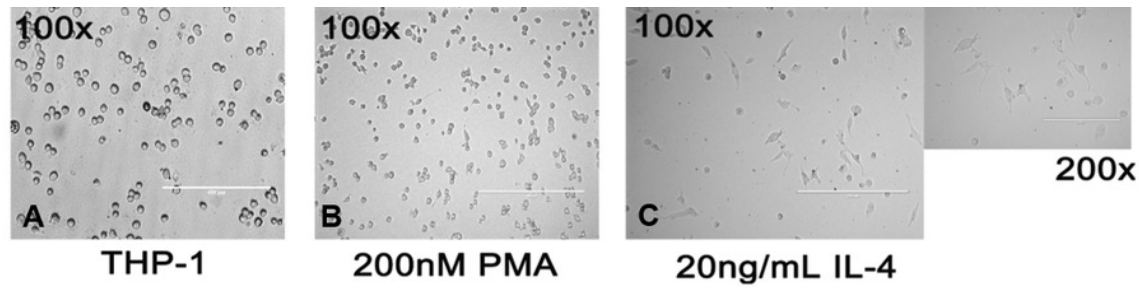
Figure 3

TAM-like PBM-derived macrophages increase the invasiveness of bladder cancer cells through CXCL8.

(A-I) For Transwell invasion assays, 5637, T24, and UM-UC-3 cells were seeded at 5.0 × 10^5 cells/well on a Matrigel-coated Transwell chamber in serum-free RPMI-1640. Serum-free RPMI-1640, CM, and CM with anti-CXCL8 neutralizing antibody were added to the Transwell chamber. The cell insert was placed on a 24-well plate containing RPMI-1640 with 10% FBS for 48 h. The invasive cells on the membrane were stained and counted. (J) The number of bladder cancer invasive cells on the membrane (1, Control; 2, CM; 3, CM with anti-CXCL8 neutralizing antibody). The results were expressed as the mean ± SD. * $p < 0.05$. (K-L) The expression level of MMP-9 in 5637, T24, UM-UC-3 was determined by (K) qRT-PCR and (L) ELISA (1, Control; 2, CM; 3, CM with anti-CXCL8 neutralizing antibody). Expression levels are expressed as the mean ± SD. * $p < 0.05$.
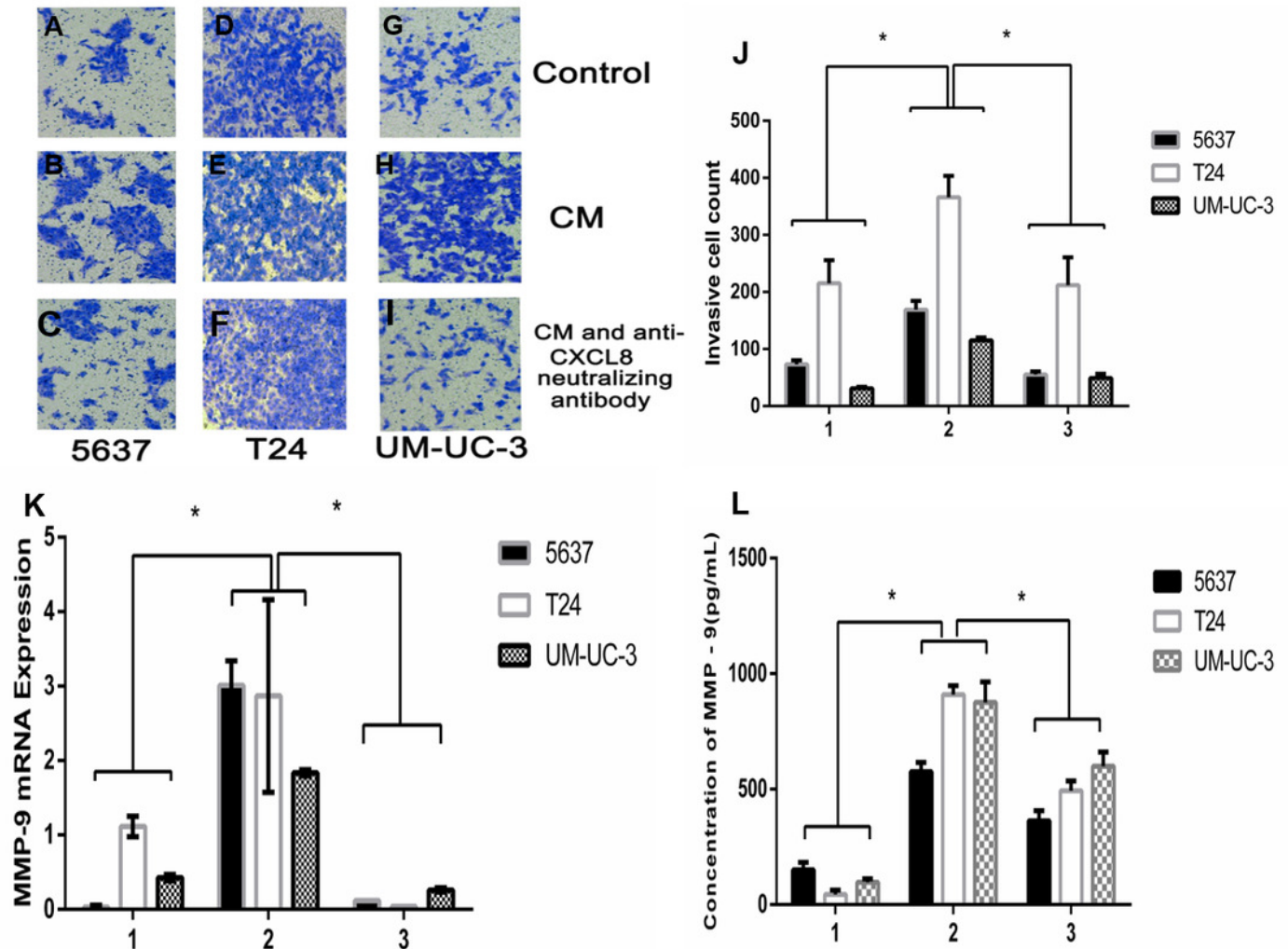
Figure 4

AM-like PBM-derived macrophagesincrease the migration of bladder cancer cells through CXCL8.

T(A-R) Three kinds of bladder cancer cell lines (5637, T24, and UM-UC-3) were treated with serum-free RPMI-1640, CM, and CM with anti-CXCL8 neutralizing antibody, respectively. Cell migration was observed at 0, 6, 12, 18, and 24 hours. Pictures taken at 0 and 24 hours. (S) Percentage of wound healing. Formula: wound healing percentage for a certain time (initial area minus area at a certain time point) to the initial area (1, Control; 2, CM; 3, CM with anti-CXCL8 neutralizing antibody). The results were expressed as the mean ± SD. * $p < 0.05$, ** $p < 0.01$. (T) The expression level of E-cadherin in 5637, T24, UM-UC-3 was determined by qRT-PCR (1, Control; 2, CM; 3, CM with anti-CXCL8 neutralizing antibody). Expression levels are expressed as the mean ± SD. *** $p < 0.001$. (U-V) 5637, T24, UM-UC-3 cells in serum-free conditions were treated with control, CM and CM with anti-CXCL8 neutralizing antibody. Western blotting was conducted with total protein extracted from bladder cancer cell lines using specific antibodies against E-cadherin and β-actin. The results are mean ± SD. *$p < 0.05$, **$p < 0.01$. (1, 2, 3 were 5637 treated with control, CM and CM with anti-CXCL8 neutralizing antibody; 4,5,6 were T24 treated with control, CM and CM with anti-CXCL8 neutralizing antibody; 7,8,9 were UM-UC-3 treated with control, CM and CM with anti-CXCL8 neutralizing antibody.)
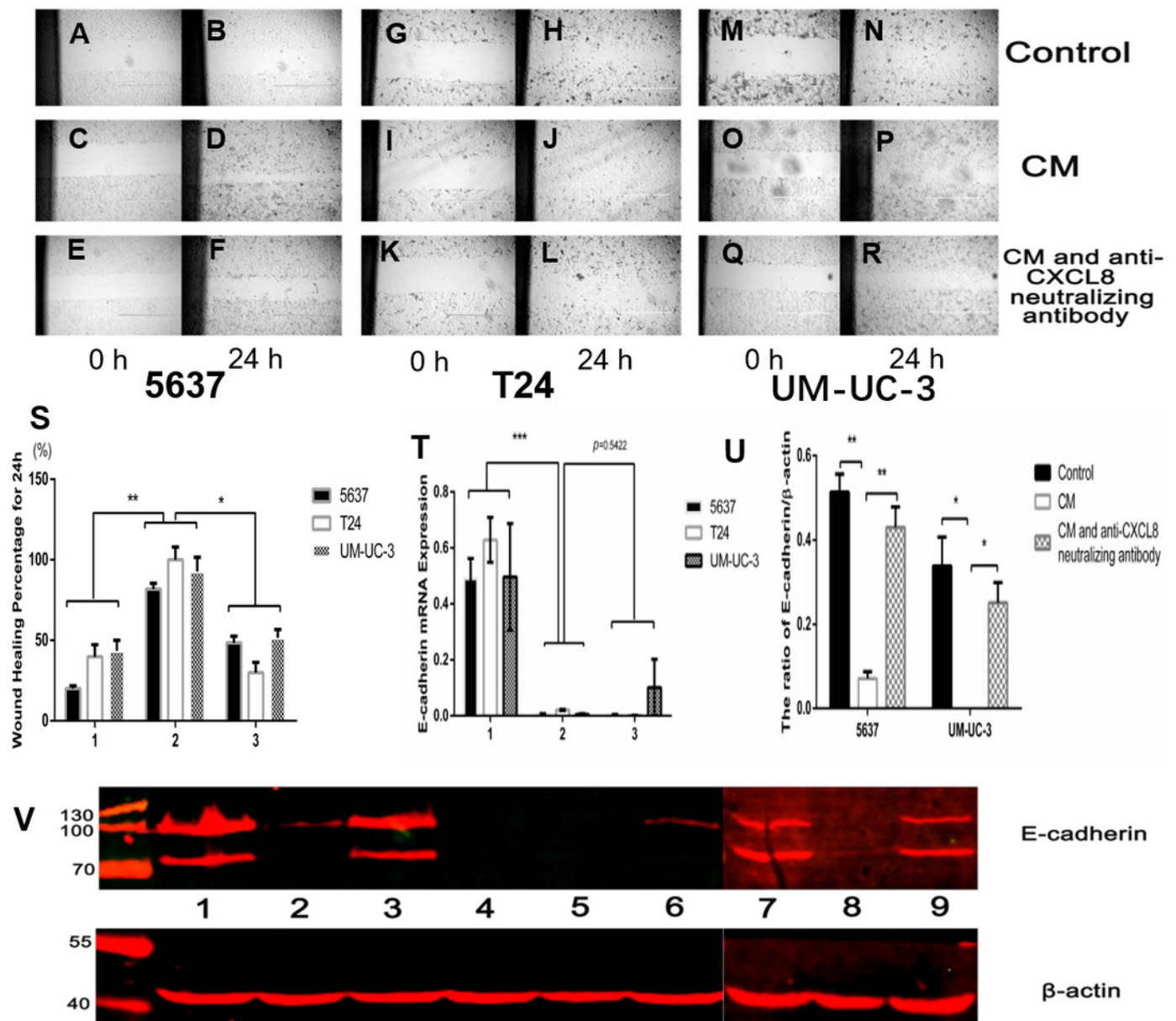
Figure 5

CXCL8-derived TAM-like PBM-derived macrophagespromote the pro-angiogenic ability of bladder cancer cells.

(A-C) For endothelial cell tube formation experiments, EA·hy926 cells were added to a Matrigel-coated 96-well. CM-C, CM-T24, and CM-T24-NA were added to wells of a 96-well plate and the tube formation of endothelial cells was observed and photographed. (D) The number of vessels and nodes formed by EA·hy926 cells was counted and plotted on the graph. The results were expressed as the mean ± SD. * $p < 0.05$, ** $p < 0.01$. (E) The expression level of VEGF in TAM-like PBM-derived macrophages was determined by qRT-PCR (1, Control; 2, CM; 3, CM with anti-CXCL8 neutralizing antibody). Expression levels are expressed as the mean ± SD. * $p < 0.05$, ** $p < 0.01$. (F-G) T24 cells were treated with serum-free medium, CM, and CM with anti-CXCL8 neutralizing antibody. The protein level of VEGF in the bladder cancer cell lines was confirmed by western blotting. Anti-VEGF and β-actin antibodies were used. The results are mean ± SD. * $p < 0.05$, ** $p < 0.01$. (1, 2, 3 were T24 treated with control, CM and CM with anti-CXCL8 neutralizing antibody.)

A CM-C **B** CM-T24 **C** CM-T24-NA

D

The number of vessels and nodes

- ■ The number of vessels
- □ The number of nodes

CM-C CM-T24 CM-T24-NA CM-C CM-T24 CM-T24-NA

E

VEGF mRNA Expression

- ■ 5637
- □ T24
- ▨ UM-UC-3

F

VEGF

β-actin

G

The ratio of VEGF/β-actin

p=0.0546