Giant cell tumor stromal cells: Osteoblast lineage-derived cells secrete IL-6 and IL-10 for M2 macrophages polarization

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Background. The giant cell tumor (GCT) is a benign tumor, which consists of three types cells: mononuclear histiocytic cells (MNHCs), multinuclear giant cells (MNGCs), and GCT stromal cells (GCTSCs). Numerous studies claim that GCTSCs have mesenchymal stem cells (MSCs) characters and play an important role in osteoclastogenesis , however, there are no research concerning about macrophage polarization among GCT, which can be regarded as an ingredient for tumor aggression. **Method.** We tested the effect of GCTSCs from three GCT samples which were collected from patients on proliferation, apoptosis and polarization of macrophage. **Result.** In this article, we verified that GCTSCs expressed MSCs markers and had higher proliferation and relative lower differentiation abilities compared with BMMSCs. What's more, we found a higher proportion of M2 macrophages among neoplasm. Co-culturing GCTSCs with macrophages resulted in prominent macrophage M2 polarization and increased the release of IL-6 (Interleukin-6) and IL-10 (Interleukin-10)from GCTSCs. In conclusion, GCTSCs, as originating from MSCs, can secret IL-6 and IL-10, which may play a significant role in macrophage M2 polarization.

1 **Giant Cell Tumor Stromal Cells: Osteoblast Lineage-Derived Cells Secret IL−6 and IL-10**

2 **for M2 Macrophages Polarization**

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Abstract

25 **Background.** The giant cell tumor (GCT) is a benign tumor, which consists of three types cells:
26 mononuclear histiocytic cells (MNHCs), multinuclear giant cells (MNGCs), and GCT stromal

27 cells (GCTSCs). Numerous studies claim that GCTSCs have mesenchymal stem cells (MSCs)
28 characters and play an important role in osteoclastogenesis, however, there are no research
29 concerning about macrophage polarization among GCT, which can be regarded as an ingredient
30 for tumor aggression.
31 **Method.** We tested the effect of GCTSCs from three GCT samples which were collected from
32 patients on proliferation, apoptosis and polarization of macrophage.
33 **Result.** In this article, we verified that GCTSCs expressed MSCs markers and had higher
34 proliferation and relative lower differentiation abilities compared with BMMSCs. What's more,
35 we found a higher proportion of M2 macrophages among neoplasm. Co-culturing GCTSCs with
36 macrophages resulted in prominent macrophage M2 polarization and increased the release of IL-
37 6 (Interleukin-6) and IL-10 (Interleukin-10)from GCTSCs . In conclusion, GCTSCs, as
38 originating from MSCs, can secret IL-6 and IL-10, which may play a significant role in
39 macrophage M2 polarization.
40 **Key words:** giant cell tumor stromal cells, macrophages, polarization, interleukin-6, interleukin-

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Introduction

44   The giant cell tumor (GCT) is a locally aggressive benign tumor which incidence accounts
45 for nearly 3–5% of primary bone neoplasm[1]. About 80% patients with GCT are aged 20 to 40.
46 The GCT induced lesions usually occur at long bones, mostly located at distal femur, proximal
47 tibia, and distal radius, sometimes at sacrum. The typical radiographic features of GCT include
48 lytic mass with cystic degeneration and intact fibrous periosteum margin[2, 3].

49   GCT consists of three mainly cells types: the mononuclear histiocytic cells (MNHCs),
50 which are driven from hematopoietic system, multinuclear giant cells (MNGCs), and GCT
51 stromal cells (GCTSCs). GCTSCs are the neoplastic component of GCT, which is regarded as a
52 promoter for accumulation, size and activity of giant cells [4-6]. It has been claimed that
53 GCTSCs originate from mesenchymal stem cells (MSCs)[5, 7] and express MSCs markers
54 CD73, CD105, CD166[7]. What's more, GCTSCs are also capable of multilineage
55 differentiation into adipocytes and osteocytes, which can secret bone matrix proteins such as type
56 I collagen, osteocalcin and bone sialoprotein[7-10].

57   However, apart from the above features, the mechanisms that how GCTSCs affect tumor
58 cell invasion and metastasis are still unclear. In view of GCT neoplasm was full filled with

59   macrophages, we speculate GCTSCs may have a connection with tumor associated macrophages

60   (TAMs)[11]. It has been evidenced that transplanting MSCs into experimental spinal cord injury

61   model may alternatively activate M2 macrophages[12]. As an anti-inflammatory factor, M2

62   macrophages regulate inflammatory responses and promote angiogenesis, tissue remodeling and

63   repair, which enable them to play a significant role in tumor aggression[13, 14].

64   Some studies have demonstrated that MSCs can convert macrophages into M2 phenotype

65   by producing IL-10[15], which has been evidenced as major cytokine inducing M2

66   polarization[16-19]. In the current study, we analyzed the expression of cytokines in BMMSCs

67   and GCTSCs, the result showed an enhanced secretion of IL-6 and IL-10 in GCTSCs while co-

68   cultured with macrophages. Considering GCTSCs originate from MSCs, it has been

69   hypothesized that GCTSCs might influence the macrophages M2 polarization in which IL-6 and

70   IL-10 play an important role. In this study, we show that GCTSCs promoted macrophages M2

71   polarization via increasing IL-6 and IL-10 secreting, which result in GCT protruding aggression.

72

73   **Materials & Methods**

74   **Clinical samples**

75   Three GCT tissue samples were collected from patients who underwent surgical resection for

76   primary GCT in Department of Orthopaedics, Xijing Hospital, the Fourth Military Medical

77   University. The patient's consent was obtained and informed consent form was signed. The use

78   of all patient-derived materials was approved by the Ethics Committee of the Fourth Military

79   Medical University (IRB-REV-2015005), and patient informed consent was obtained

80   individually.

81   **Cell culture**

82   Primary GCT stromal cells (GCTSCs) were isolated from fresh GCT. The neoplasm was minced

83   in small pieces and digested with 200 U/ml collagenase (Gibco BRL, Karlsruhe, Germany) for 1

84   h at 37°C. Cells were collected by centrifugation, washed twice in PBS and cultured with alpha

85   minimum essential medium (α-MEM, Gibco BRL, Gaithersburg, MD, USA) containing 10%

86   FBS (Hangzhou Sijiqing Biological Engineering Materials Co., Ltd. Zhejiang, China) and 100

87   U/ml penicillin and 100 mg/ml streptomycin (Invitrogen Life Technology, Carlsbad, CA, USA).

88   The cells were kept in culture at 37°C, 5% CO_2, and the media was changed every 3 days

89   thereafter until cells reached 70% confluency. Adherent GCTSCs were harvested using 0.25%

90   Trypsin-EDTA (Invitrogen Life Technology, Carlsbad, CA, USA). Normal MSCs were obtained

91   from the bone marrow of C57 mice as described previously (Soleimani M and Nadri S 2009).

92   Human monocytes were isolated from the peripheral blood of normal human volunteers (Blood

93   donors in Blood Transfusion Department of Xijing Hospital) using human monocyte isolation kit

94   II (Miltenyi Biotech, Teterow, Germany). Purified CD14$^+$ monocytes were plated into 6-well cell

95   culture plates at a concentration of 1×10^6 per well in RPMI 1640 media supplemented with 10%

96   FBS.

97   **Animal experiments**

98   Immunocompromised mice (NMRI-nu/nu) were purchased from Beijing Vital River Laboratory,

99   Beijing, China. All mice were housed five per cage under standard laboratory conditions at 12 h light

100 /dark cycles, and 22℃, with free access to food and water. Mice were anesthetized by intraperitoneal

101 injection of pentobarbital (30mg/kg) and implanted with cells. After 35 days of implantation, the mice

102 were decapitated and killed, the de novo bone area was taken. Animal experiments in this study was

103 authorized by the Animal Care Committee of Fourth Military Medical University (2016-kq-054),

104 and all experimental protocols were performed with the approval of the Fourth Military Medical

105 University.

106 **Histology and Immunohistochemistry Staining**

107 The neoplasm specimens were fixed in 4% paraformaldehyde overnight and then embedded in

108 paraffin or in the optimal cutting temperature compound. Hematoxylin and eosin (HE) staining

109 was used for general observation. Surface markers of GCTSCs and macrophages were stained

110 with CD105 and CD14 antibodies (eBiosciences, San Diego, CA, USA) overnight at 4°C,

111 respectively. When they were washed 3 times, peroxidase conjugated goat anti-rabbit secondary

112 antibody (Dako, Denmark) was added. Envision Detection System kit (DAKO, Denmark) was

113 used after the nuclear stained by hematoxylin. Color development was triggered using Di-Amino

114 Benzidine (DAB, Calbiochem, Germany).

115 **Immunofluorescent Staining**

116 BMMSCs and GCTSCs were washed with PBS and fixed with 4% paraformaldehyde for 15 min,

117 and then were incubated with antibodies CD146, CD90, CD73, STRO-1 and SSEA-4, as well as

118 antibodies against hematopoietic cell markes CD14 and CD34 and CD45 (All from BD

119 Biosciences, San Diego, CA, USA). The positively stained cells were detected by a laser

120 scanning confocal microscope (Olympus FluoView FV 1000, Tokyo, Japan).

121 **Flow Cytometry Analysis**

122 To confirm mesenchymal stem cells character, we used flow cytometric analysis to show that

123 GCTSCs were positive for CD90, CD105, and CD29, and negative for CD14, CD34 and CD45

124 (All from BD Biosciences, San Diego, CA, USA). Ki-67 staining was used to confirm the

125 proliferation of macrophages after being co-cultured with GCTSCs. Apoptotic cells were

126 examined using Annexin V-FITC/7AAD Apoptosis Detection Kit (Roche, Basel, Switzerland).

127 Cells were stained with Annexin V-FITC and 7AAD for 15 min, both of which were in the dark

128 at room temperature. After washing by PBS, immunofluorescent-stained cells were analyzed on

129 FACS (Becton-Dickinson) can using Consort 30 software.

130 **BrdU test**

131 BMMSCs and GCTSCs were labeled with 5-bromodeoxyuridine (BrdU, sigma) 10 umol·L^{-1} for

132 24 h. After the cells were washed three times with PBS and fixed with 4% paraformaldehyde for

133 15 min, the incorporated BrdU was detected by mouse anti-BrdU monoclonal antibody

134 conjugated with peroxidase (Abcam). The color reaction was parallel with the number of

135 proliferating cells.

136 **Osteogenic and Adipogenic Differentiation**

137 BMMSCs and GCTSCs were incubated with osteogenic medium (100 nmol·L^{-1} dexamethasone,

138 50 mg·mL^{-1} ascorbic acid and 1 mmol·L^{-1} b-glycerophosphate, Sigma) for 21 d according to the

139 manufacturer´s instructions. Cells were fixed with 60% isopropanol and stained with 1%

140 Alizarin Red (Sigma) for osteogenic differentiation assay. BMMSCs and GCTSCs were also

141 cultured with adipogenic medium (0.5 mmol·L^{-1} methylisobutylxanthine, 0.5 mmol·L^{-1}

142 hydrocortisone and 60 mmol·L^{-1} indomethacin, Sigma) for 14 d. Intracellular lipid droplet was

143 detected by staining with Oil Red O solution.

144 **In vivo bone formation assay**

145 5×10^6 cells were combined with 40 mg hydroxyapatite/tricalcium phosphate (HA/TCP) and

146 implanted at the back of immunocompromised mice (NMRI-nu/nu). After 35 days the mice were

147 euthanized and the de novo bone area was stained by HE. The sections were analyzed by Image-

148 Pro Plus software.

149 **Co-cultures**

150 The purified human blood monocytes were indirectly co-cultured with GCTSCs as well as

151 BMMSCs for 14 d. The monocytes (2×10^5cells) were cultured in 24 well plates and GCTSCs or

152   BMMSCs (2×10^4 cells) were cultured in the cell-culture insert (pore size: 0.4 pm) of the same

153   well (Falcon Inc.).

Antibody-based protein array

155   The assay was performed following the manufacturer's instructions (RayBio). Briefly, samples

156   with equal amounts of total proteins were incubated overnight at $4℃$. After washing, the

157   membranes were incubated with Biotinylated Antibody Cocktail followed by second wash.

158   Incubate with HRP-Streptavidin overnight at $4℃$ and wash again. Chemiluminescence was used

159   for signal detection, which was analyzed with Quantity One image analysis software (Molecular

160   Imager FX, Bio-Rad Laboratories).

Quantitative RT-PCR

162   Total RNA was isolated from BMMSCs and GCTSCs by using RNAiso plus (TaKaRa, Tokyo,

163   Japan) according to the manufacturer's instructions. The mRNA was reversed to complementary

164   DNA, and quantitative real time -PCR (qRT-PCR) detection was carried out by Prime Script TM

165   RT master mix (TaKaRa, RR036A) and SYBR Premix Ex TaqTMII (TaKaRa). A CFX96

166   Trademark Real-time PCR detection system (Bio-Rad, Richmond, CA, USA) was used for the

167   detection. The forward and reverse primers were used to measure IL-6 (F:

168   AGTGAGGAACAAGCCAGAGC,R: AGCTGCGCAGAATGAGATGA) and IL-10 (F:

169   ACCTGCCTAACATGCTTCGAG, R: TGGGTCTTGGTTCTCAGCTTG) gene expression, and

170   target gene expression levels were normalized to that of GAPDH (F:

171   TGACATCAAGAAGGTGGTGAAGC, R: GGAAGAATGGGAGTTGCTGTTG).

Statistical Analysis

173   GraphPad Prism software (Graph-Pad Software, Inc., USA) was used for statistical analysis. All

174   data are presented as mean±s.d and the measurements were performed on three different GCT

175   patient samples (n = 3). Unpaired, two-tailed Student's t tests for comparisons between two

176   groups and one-way analysis of variance (ANOVA) with Bonferroni for multiple comparisons

177   were applied. Each experiment was repeated at least three times. P value < 0.05 was considered

178   to be statistically significant.

179

Results

CD14 and CD105 cells exist in Gaint cell tumor

182   We harvested Giant cell tumor (GCT) samples from three patients with two females (46 and

183   41 years old) and one male (21 years old). The lesions in which were tibia, ilium and both

184   terminal of the tibia and femur. All of these three patients presented a common radiologic

185   feature: a lytic and well-defined lesion without sclerotic margin **(Figure 1a)**. HE staining showed

186   GCT consisted of three kinds of cells: multinucleated giant cells, mononuclear histocytic cells

187   and giant cell tumor stromal cells (GCTSCs) with a spindle morphology. Immunohistochemistry

188   staining verified GCT cells contained of CD14 positive and CD105 positive cells, which

189   represented a macrophage-like and mesenchymal-like features respectively **(Figure 1b)**.

190   Immunofluorescence staining of the GCT indicated STRO-1 positive and CD14 positive cells in

191   the samples and these two kinds of cells were not overlapped.**(Figure 1c)**.

192

193   **GCTSCs showed increased proliferation and decreased differentiation characteristics**

194   It had been widely proved that giant cell tumor stromal cells (GCTSCs) were the neoplastic

195   component of GCT and had similar functions with mesenchymal stem cells. We isolated the cells

196   from neoplasm and compared different bio-markers (CD90, CD105, CD29, CD14, CD34, CD45)

197   between three patients' GCTSCs and normal bone marrow MSCs. Flow cytometer analysis

198   indicated that both GCTSCs and BMMSCs had a similar specific expression of CD90, CD105

199   and CD29 and negative expression of CD14, CD34 or CD45 **(Figure 2a)**. Immunofluorescence

200   results also confirmed the positive expressions of STRO-1, SSEA-4, CD90 and CD146 in

201   GCTSCs, which validated that GCTSCs had similar immune profile with BMMSCs **(Figure 2b)**.

202   To characterize the proliferation property of GCTSCs, we performed serial implantation of

203   GCTSCs. Brdu measurement indicated that all of three GCTSCs had a significantly higher

204   proportion of Brdu-positive cells than BMMSCs (P<0.01). After subcutaneously implantation of

205   GCTSCs into immunocompromised mice, we found that re-GCTSCs collected from implants

206   still showed higher proliferation than re-BMMSCs. **(Figure 2c)**. These results indicated GCTSCs

207   had mesenchymal stem cell like properties and had an obviously higher proliferative activity

208   than BMMSCs.

209   In order to examine the differentiative capacity of GCTSCs into osteoblast and adipocyte,

210   we induced the GCTSCs with osteogenic and adipogenic induction medium respectively.

211   Alizarin Red staining verified that the total mineralized area in GCTSCs was 0.11 compared with

212   0.25 in BMMSCs (2.27-fold higher, P=0.056) **(Figure 2d)**. Oil-Red O stained cells represented

213   the adipocytes, the proportion of which in GCTSCs was 2.22% compared with 32.44% in
214   BMMSCs (14.61-fold higher, P<0.01) **(Figure 2e)**. GCTSCs or BMMSCs combined HA/TCP
215   were subcutaneously implanted into immunocompromised mice, and a clearly bone formation
216   area of BMMSC implantation was founded, the total *de novo* bone area was about 0.12%,
217   compared with much smaller spots about 0.04% among GCTSC implantation (P<0.001) **(Figure
218   2f)**. These results indicated that GCTSCs indeed had bone and adipose differentiation capacities
219   to some extent in vitro as well as in vivo, but which were much weaker than BMMSCs.

220

221   **GCTSCs play no role in proliferation or apoptosis of CD14 cells**

222   Many researches had pronounced that GCTSCs played a significant role in giant cells
223   formation, osteolysis as well as aggression, but there were rarely researches to explore the
224   influence of GCTSCs on the proliferation or apoptosis of CD14 cell. Co-culturing macrophage
225   cells with GCTSCs and BMMSCs using transwell for 7 days, 14 days and 21 days respectively
226   and the amount of macrophage cells was counted. The results of different co-culturing period
227   with GCTSCs or BMMSCs exhibited no significance correlation **(Figure 3a)**. The proportion of
228   Ki67 cells, as an indication of proliferative ability, among normal macrophages and co-cultured
229   with BMMSCs as well as GCTSCs are 3.83%, 2.57% and 4.72% (P>0.05) **(Figure 3b, d)**, which
230   indicated both GCTSCs and BMMSCs didn't alter the proliferative ability of CD 14 positive
231   macrophages.

232   Moreover, we detected the Annexin V+7-ADD+ macrophages after co-cultured with
233   BMMSCs or GCTSCs. BMMSCs had a prominent tendency in inducing macrophage apoptosis,
234   the proportion of which was approximately 3.65% (P=0.01), however, co-culture with GCTSCs
235   hadn't resulted in any enhancement of macrophage apoptosis, which were 1.25%, 3.65% and 1%
236   (BMMSCs vs Macrophage, P<0.05. GCTSCs vs Macrophage, P>0.05), respectively**(Figure 3c,
237   d)**. These data confirmed that BMMSCs could induce the apoptosis of macrophage but not the
238   GCTSCs. However, the proportion of apoptosis cells induced by BMMSCs was very low.

239

240   **GCTSCs promote the polarization into M2 phenotype of macrophage via increasing IL-6
241   and IL-10 release**

242   It had been wildly accepted the polarization of macrophage into M2 phenotype was a
243   contribution for tumor propagation and angiogenesis. In this research, we found a numerous

244   proportion of M2 phenotype macrophages among the neoplasm (85.05%) compared with normal

245   macrophages (61.15%) (P<0.05) **(Figure 4a)**. By co-culture the macrophages with GCTSCs as

246   well as BMMSCs, it aroused a significant (11.0%, 11.6%, 19.1%) polarization of M2

247   macrophages in GCTSCs and 8.6% in BMMSCs **(Figure 4b)**. These results verified that,

248   compared with BMMSCs, GCTSCs could promote the macrophages into M2 phenotype.

249   Human cytokine antibody array can semi-quantitative detected 42 human proteins

250   expression using 2-D densitometry. By analyzing the culture supernatant of BMMSCs and

251   GCTSCs, it was clarified that GCTSCs had a distinctive extraction in the spot of IL-6 and IL-10

252   **(Figure 4c)**. q-PCR analysis verified that GCTSCs had a much higher expression of IL-6 and IL-

253   10 compared with BMMSCs (P<0.05) (Figu**re 4d)**. These results illustrated that GCTSCs

254   expressed and secreted a large amount of IL-6 and IL-10, which might induce the polarization

255   into M2 phenotype macrophages.

256

Discussion

258   It has been wildly accepted that GCTSCs are the neoplasms component of the bone and

259   express mesenchymal stem cell (MSCs) markers[5, 7, 9]. In this study, we confirm GCTSCs

260   express MSCs-like markers STRO-1, SSEA-4, CD90, CD146, CD105, CD29, which are in

261   accordance with BMMSCs[9, 20]. And are the neoplastic component, which have a prominent

262   proliferative activity that can be stained in Brdu measurement. Others reported that GCTSCs

263   expressed alkaline phosphatase, osteocalcin and Cbfa1, equipping a multi-differentiation ability

264   to form mineralized node and adipose not only in vitro, but also in vivo[10, 21]. In the present

265   results, GCTSCs could differentiated into osteoblasts and adipocytes, which was in accordance

266   with the previous findings. However, comparing with the BMMSCs, GCTSCs have weaker

267   differentiation capacities.

268   Previous researches had reported that tumor cells could recruiting circulating monocytes,

269   osteoclast precursor cell or even osteoclasts by producing transforming growth factor-1 (TGF-

270   β1) and monocyte chemoattractant protein 1 (MCP-1) [22]. Jenkins SJ *et al* discovered that IL-4

271   and filarial nematode infection can induce resident macrophages proliferation without recruiting

272   blood cells [23]. In order to confirm if GCTSCs can alter macrophages proliferative ability, we

273   co-cultured macrophage cells with GCTSCs and BMMSCs using transwell. The Ki67+ cells

274   exhibit a similar proportion among control, GCTSCs and BMMSCs groups, which means

275 GCTSCs don't induce macrophages proliferation. Simultaneously, the proportion of Annexin

276 V+7ADD+ macrophages was increased after co-culture with BMMSCs, indicates that BMMSCs

277 can induce more macrophage cells apoptosis than GCTSCs.

278 Nevertheless, no former research has focused on the relationship between GCTSCs and

279 macrophages polarization. We for the first time discovered a numerous proportion of M2

280 phenotype macrophage cells among the neoplasm (85.05%) compared with normal macrophages

281 (63.15%) (P<0.05). Then, we co-cultured GCTSCs with macrophages and it exhibited a higher

282 proportion of M2 macrophages than BMMSCs. By analyzing the culture supernatant of

283 BMMSCs and GCTSCs with human cytokine antibody array, we found a higher IL-6 and IL-10

284 secretion, consistent with the array data, an increased mRNA expression was found in IL-6 and

285 IL-10. We speculated the cytokines secreted by GCTSCs may engage in macrophage M2

286 polarization.

287 It has been clarified that MSCs can alter the phenotype of macrophages from M1 type into a

288 M2 population, which are indicative of pro-tumor activity [14, 24], but there are no articles that

289 analyze macrophage phenotype among GCT. Former researchers had claimed that

290 immunoregulation cytokines like IL-4, IL-10 and IL-13 induced the M2 phenotype polarization

291 [19]. However fewer articles reported that IL-6 may also be a novel cytokine that plays an

292 important role in M2 macrophage polarization[18, 25]. IL-6 can enhances IL-4R-α expression

293 and augments the phosphorylation of STAT6, as IL-4-STAT6 axis is one of the most potent

294 inducer of M2 polarization[18]. As we have detected both IL-6 and IL-10 high secretion of

295 GCTSCs, it's interested for us to explore the connection between GCTSCs and M2

296 macrophages. In this article, there are still many phenomenon need further explore. (a) Whether

297 the M2 polarization on account of GCTSCs play a significant role in GCT invasion and

298 metastasis. (b) The exact mechanisms of IL-6 and IL-10 in inducing M2 polarization among

299 GCT are still unknown. In conclusion, we first confirm that GCTSCs are belong to osteoblast

300 lineage and have a enhanced proliferative ability as well as a deduced differentiative ability.

301 Although numerous population of macrophages emerge in giant cell tumor, GCTSCs have none

302 influence on its proliferation of apoptosis. IL-6 and IL-10 are found expressed highly among

303 GCTSCs, while they are speculated to play a significant role in M2 phenotype macrophages.

304

305 **Conclusions**

306   Our research proves that, GCTSCs expressed MSCs markers and had higher proliferation

307   and relative lower differentiation abilities compared with BMMSCs. Co-culturing GCTSCs with

308   macrophages promoting macrophages polarization into M2 phenotype. At the same time, the

309   level of IL-6 and IL-10 released from GCTSCs was increased. In short GCTSCs contribution for

310   tumor proliferation via promoting macrophages M2 phenotype polarization by improving IL-6

311   and IL-10 secretion.

312

313   **Figure 1. CD14 and CD105 cells are observed in Gaint cell tumor by tissue staining.** (a) X-

314   Ray indicates the lesion area of three GCT patients are tibia, ilium and both terminal of the tibia

315   and femur, respectively. (b) HE staining indicates that the neoplasm is consist of three categories

316   cells: multinucleated giant cells, mononuclear histiocytic cells and GCTSCs. Scale bar = 50 μm.

317   (c) Immunofluorescent staining exhibits the STRO-1$^+$ cells (green) have a different distribution

318   with CD14$^+$ cells (red), which means that they belong to two distinguished lineages. Scale

319   bar = 10 μm.

320

321   **Figure 2. GCTSCs showed increased proliferation and decreased differentiation**

322   **characteristics. (a)** Flow cytometry analysis indicates that both of BMMSCs and GCTSC1,

323   GCTSC2, GCTSC3 have common expressed MSCs markers CD90, CD105, CD29, and rarely

324   express hematopoietic lineage markers CD14, CD34, CD45. **(b)** Immunofluorescence staining

325   verified that GCTSCs express STRO-1, SSEA-4, CD90, CD146, CD73 but not hematopoietic

326   markers CD45, CD34 or CD14. Scale bar = 10 μm. **(c)** Proliferation ability of both BMMSCs

327   and GCTSCs. Brdu measurement indicates the proliferating cells. Single-colony-derived MSCs

328   and GCTSCs, respectively, were expanded to 2×10^6 cells and subsequently implanted into

329   immunocompromised mice with the use of geofoams as a carrier. The proliferating cells in

330   GCTSCs1 (P<0.01) and GCTSCs2 (P<0.05) are much more than BMMSCs. However, the

331   GCTSCs3 after implantation shows a similar proliferation ability. **(d. e)** Osteogenicand

332   adipogenic differentiation of BMMSCs and GCTSCs *in vitro*. **(f)** *de novo* bone formation of

333   BMMSCs and GCTSCs *in vivo*.

334

335   **Figure 3. GCTSCs play no role in proliferation or apoptosis of CD14 cells. (a)** The

336   macrophages number co-cultured with BMMSCs and GCTSCs were compared in 7, 14 and 21

337   days. It shows that the both BMMSCs and GCTSCs have a rare effect on macrophages cells

338   number. **(b)** Ki67 expression in macrophages co-culturing with different types of cells shows a

339   similar level (P>0.05), which indicates that both BMMSCs and GCTSCs don't have a

340   promotion for macrophages proliferation. **(c)** The apoptosis of macrophages co-culturing with

341   different types of cells shows that BMMSCs (P<0.001) have a prominent tendence to induce

342   macrophages apoptosis, compared with GCTSCs (P>0.05). **(d)** Representative flow cytometry

343   analysis of macrophages proliferation and apoptosis co-cultured with BMMSCs and GCTSCs.

344

345   **Figure 4. GCTSCs promote the polarization into M2 phenotype of macrophage via**

346   **increasing IL-6 and IL-10 release. (a)** The proportion of CD206$^+$ cells among CD14$^+$ cells.

347   CD206 is a marker of M2 macrophages and it consists 85.05% of macrophages in GCT

348   compared with 61.15% in blood (P<0.05). **(b)** CD206$^+$ cells proportion in GCT CD14$^+$ cells and

349   co-cultured with BMMSC, GCTSC1, GCTSC2 and GCTSC3. (GCT CD14$^+$ cell vs. GCT CD14$^+$

350   cell +BMMSCs, P=0.483. GCT CD14$^+$cell vs. GCT CD14$^+$ cell +GCTSCs1, P=0.026. GCT

351   CD14$^+$cell vs. GCT CD14$^+$ cell +GCTSCs2, P=0.044. GCT CD14$^+$cell vs. GCT CD14$^+$ cell

352   +GCTSCs3, P<0.001**). (c)** Human cytokine antibody array analysis of BMMSCs and GCTSCs.

353   Three independent experiments obtained a similar result and one of them are shown. The

354   expression of IL-6 and IL-10 of GCTSCs is much higher compared with BMMSCs

355   (POS=Positive Control Spot, NEG=Negative Control Spot, BLANK=Blank Spot). **(d)** mRNA

356   expression level of IL-6 and IL-10 among BMMSCs and GCTSCs. The expression of IL-6 and

357   IL-10 of GCTSCs is much higher compared with BMMSCs (*P<0.05, ***P<0.001).

358

359   **Figure 5. Schematic diagram of this study.** Schematic illustration of GCTSCs exist in giant

360   cell tumor, and release IL-6 and IL-10 to promote macrophages turning into M2 polarization

361   phenotype.

362

363   **Compliance with Ethical Statements**

364   **Conflict of Interest** The authors declare that they have no conflicts of interest.

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369   **Informed consent** Informed consent was obtained from all individual participants included in

370   the study.

371

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Figure 1

CD14 and CD105 cells are observed in Gaint cell tumor by tissue staining

(a) X-Ray indicates the lesion area of three GCT patients are tibia, ilium and both terminal of the tibia and femur, respectively. (b) HE staining indicates that the neoplasm is consist of three categories cells: multinucleated giant cells, mononuclear histiocytic cells and GCTSCs. Scale bar = 50μm. (c) Immunofluorescent staining exhibits the STRO-1$^+$ cells (green) have a different distribution with CD14$^+$ cells (red), which means that they belong to two distinguished lineages. Scale bar = 10μm.
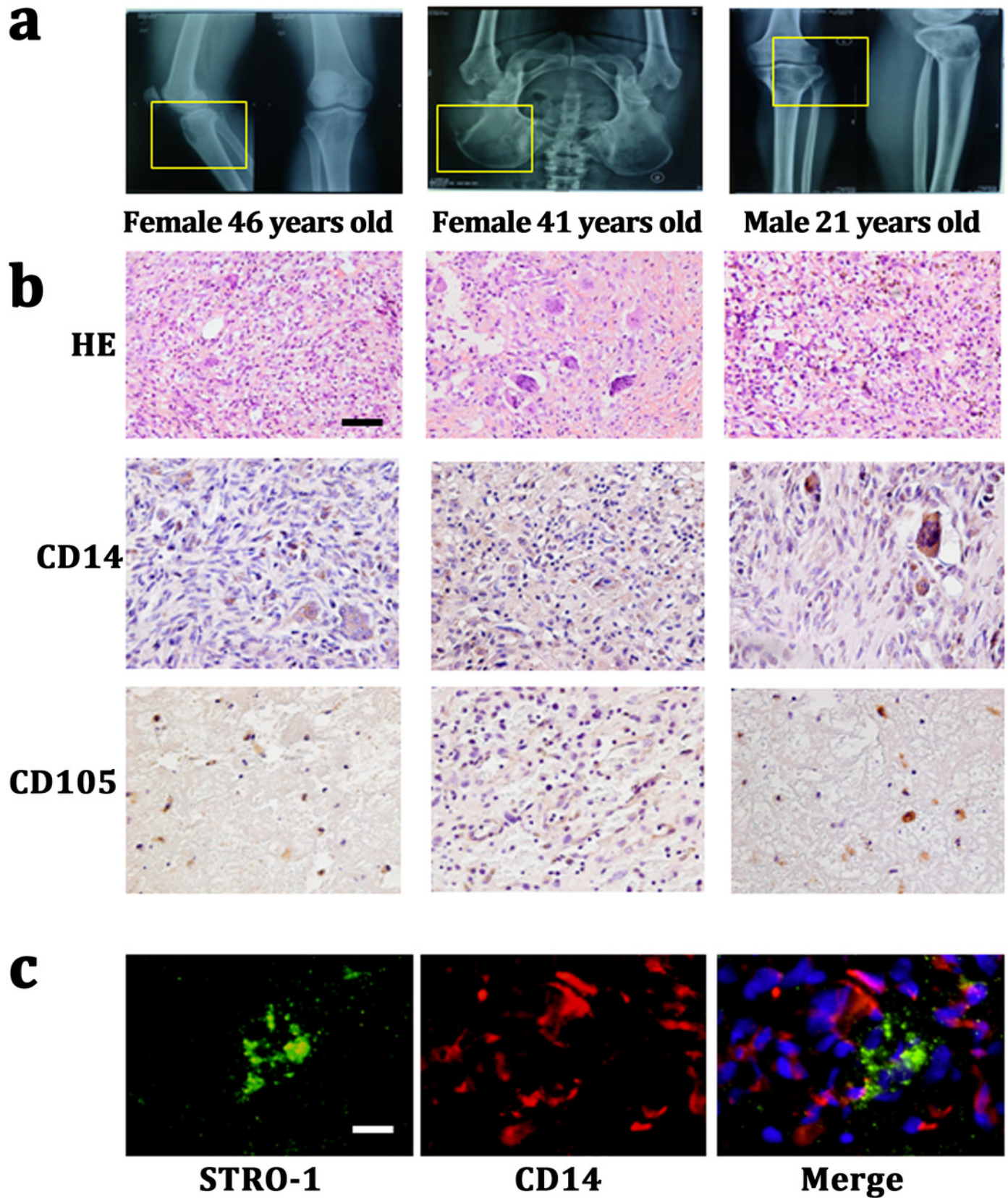
Figure 2

GCTSCs showed increased proliferation and decreased differentiation characteristics.

(a) Flow cytometry analysis indicates that both of BMMSCs and GCTSC1, GCTSC2, GCTSC3 have common expressed MSCs markers CD90, CD105, CD29, and rarely express hematopoietic lineage markers CD14, CD34, CD45. **(b)** Immunofluorescence staining verified that GCTSCs express STRO-1, SSEA-4, CD90, CD146, CD73 but not hematopoietic markers CD45, CD34 or CD14. Scale bar = 10μm. **(c)** Proliferation ability of both BMMSCs and GCTSCs. Brdu measurement indicates the proliferating cells. Single-colony-derived MSCs and GCTSCs, respectively, were expanded to 2×10^6 cells and subsequently implanted into immunocompromised mice with the use of geofoams as a carrier. The proliferating cells in GCTSCs1 (P<0.01) and GCTSCs2 (P<0.05) are much more than BMMSCs. However, the GCTSCs3 after implantation shows a similar proliferation ability. **(d. e)** Osteogenicand adipogenic differentiation of BMMSCs and GCTSCs *in vitro.* **(f)** *de novo* bone formation of BMMSCs and GCTSCs *in vivo.*
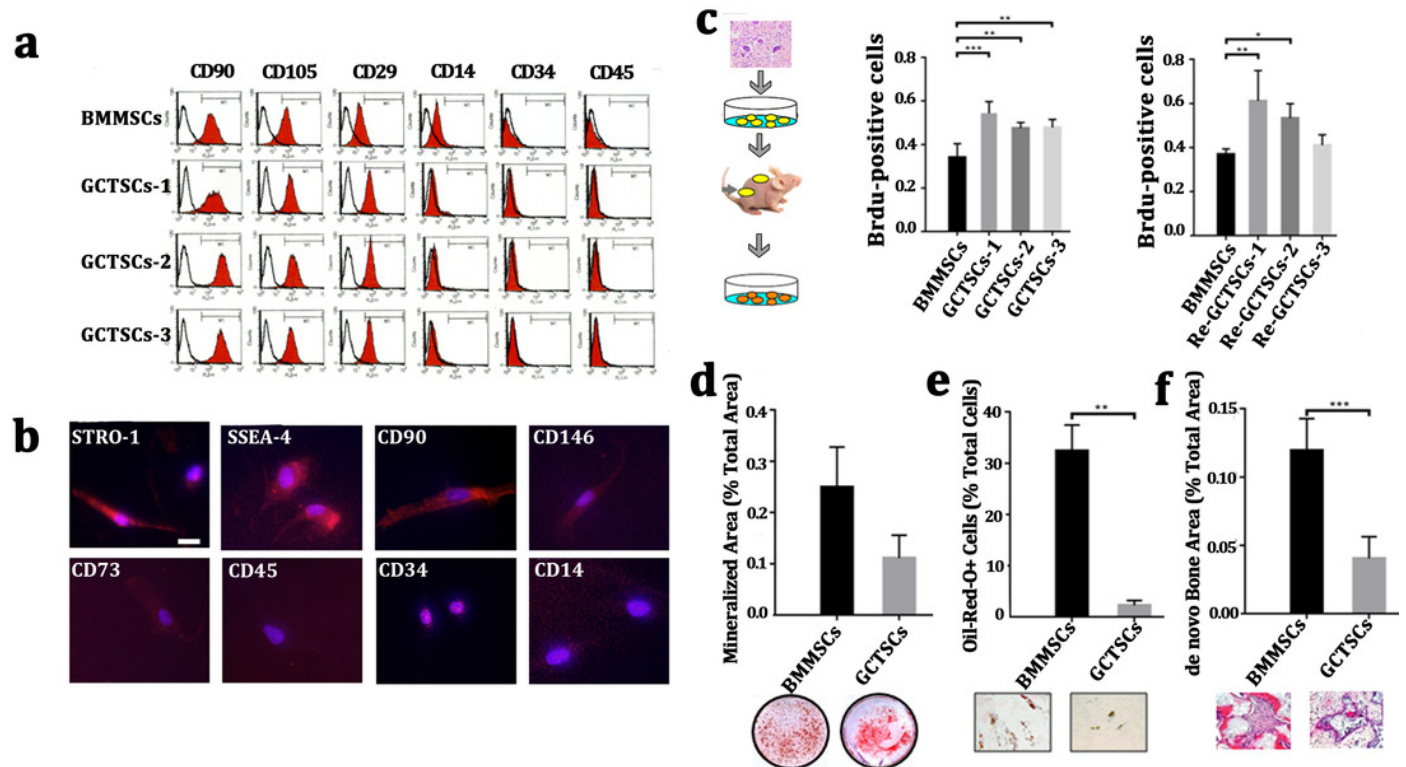
Figure 3

GCTSCs play no role in proliferation or apoptosis of CD14 cells.

(a) The macrophages number co-cultured with BMMSCs and GCTSCs were compared in 7, 14 and 21 days. It shows that the both BMMSCs and GCTSCs have a rare effect on macrophages cells number. **(b)** Ki67 expression in macrophages co-culturing with different types of cells shows a similar level (P>0.05), which indicates that both BMMSCs and GCTSCs don't have a promotion for macrophages proliferation. **(c)** The apoptosis of macrophages co-culturing with different types of cells shows that BMMSCs (P<0.001) have a prominent tendence to induce macrophages apoptosis, compared with GCTSCs (P>0.05). **(d)** Representative flow cytometry analysis of macrophages proliferation and apoptosis co-cultured with BMMSCs and GCTSCs.
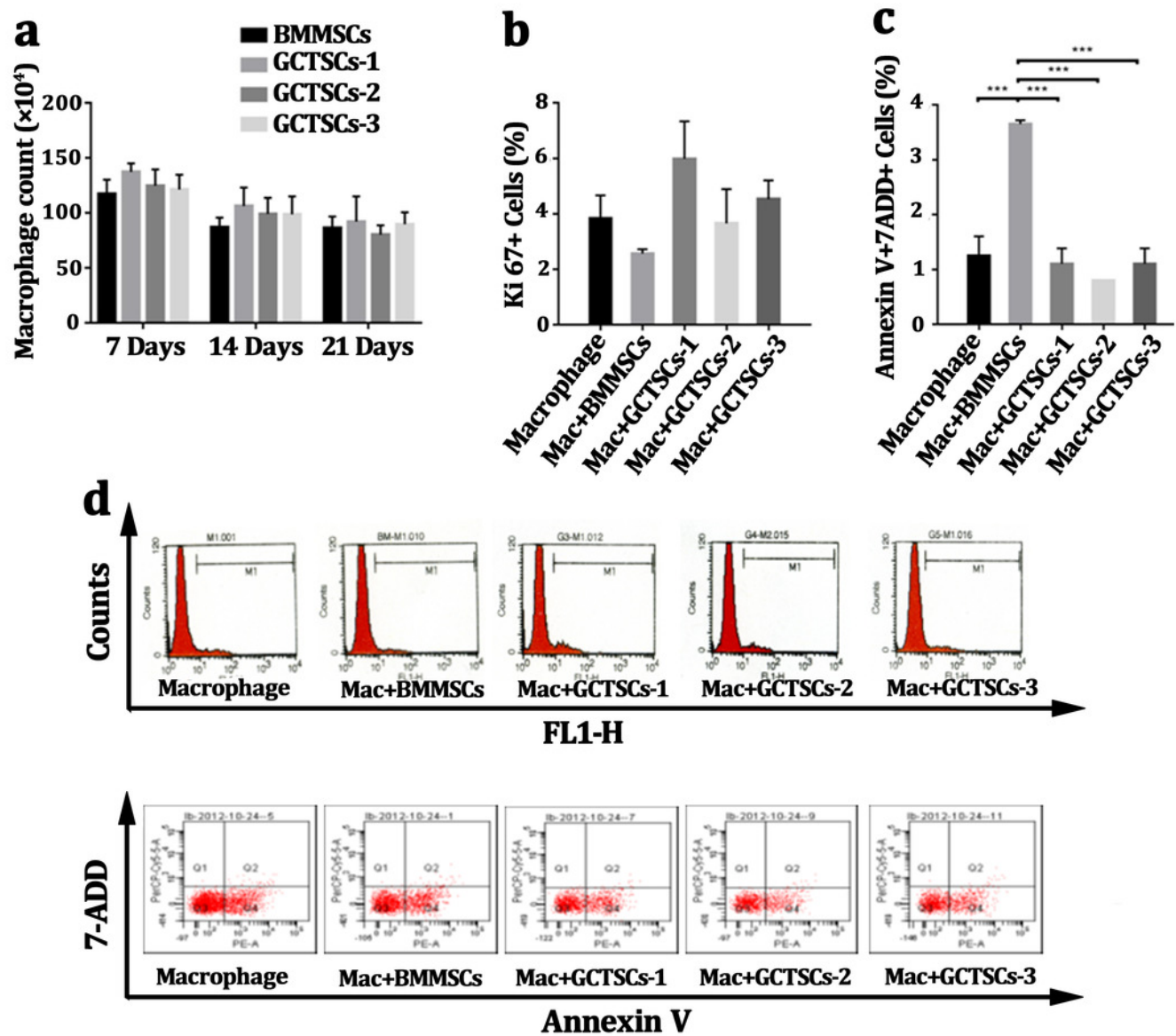
Figure 4

GCTSCs promote the polarization into M2 phenotype of macrophage via increasing IL-6 and IL-10 release.

(a) The proportion of CD206$^+$ cells among CD14$^+$ cells. CD206 is a marker of M2 macrophages and it consists 85.05% of macrophages in GCT compared with 61.15% in blood (P<0.05). **(b)** CD206$^+$ cells proportion in GCT CD14$^+$ cells and co-cultured with BMMSC, GCTSC1, GCTSC2 and GCTSC3. (GCT CD14$^+$ cell vs. GCT CD14$^+$ cell +BMMSCs, P=0.483. GCT CD14$^+$cell vs. GCT CD14$^+$ cell +GCTSCs1, P=0.026. GCT CD14$^+$cell vs. GCT CD14$^+$ cell +GCTSCs2, P=0.044. GCT CD14$^+$cell vs. GCT CD14$^+$ cell +GCTSCs3, P<0.001)**. (c)** Human cytokine antibody array analysis of BMMSCs and GCTSCs. Three independent experiments obtained a similar result and one of them are shown. The expression of IL-6 and IL-10 of GCTSCs is much higher compared with BMMSCs (POS=Positive Control Spot, NEG=Negative Control Spot, BLANK=Blank Spot). **(d)** mRNA expression level of IL-6 and IL-10 among BMMSCs and GCTSCs. The expression of IL-6 and IL-10 of GCTSCs is much higher compared with BMMSCs (*P<0.05, ***P<0.001).

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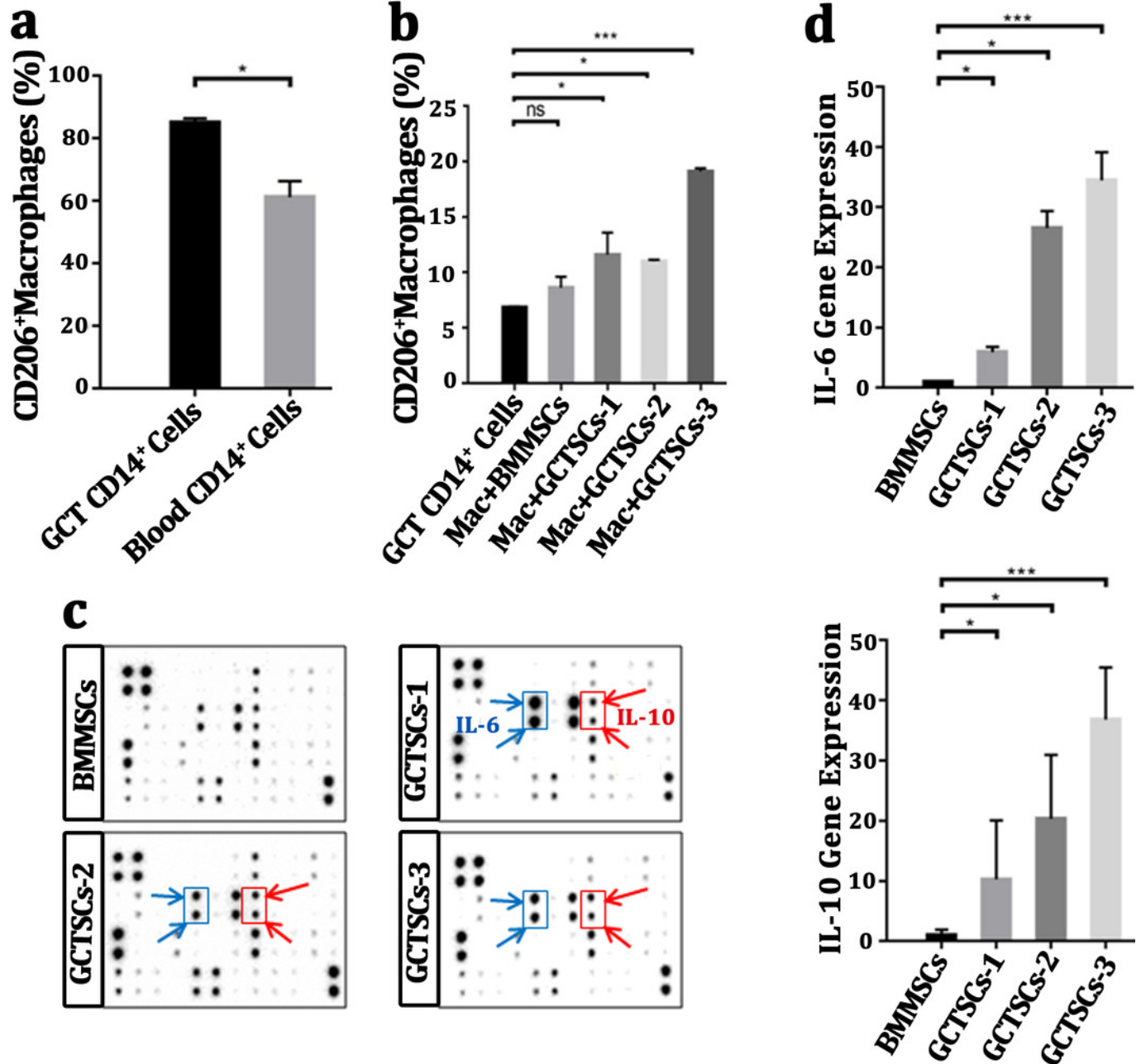
Figure 5

Schematic diagram of this study.

Schematic illustration of GCTSCs exist in giant cell tumor, and release IL-6 and IL-10 to promote macrophages turning into M2 polarization phenotype.