

Original Article

Role of Stat3 Activation in Cell-Cell Interaction between B-Cell Lymphoma and Macrophages : The *in vitro* Study

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Recently, tumor-associated macrophages (TAMs) have been of interest because of their protumoral functions. In patients with malignant lymphoma, an increased number of alternatively activated (M2) macrophages is closely associated with poor clinical prognosis. Signal transducer and activator of transcription 3 (Stat3) is an important molecule related to tumor development. Previously we demonstrated that Stat3 activation in primary central nervous system lymphoma cells was significantly associated with the number of M2 TAMs present. Here we report that direct contact with macrophages in culture was required for proliferation of B-cell lymphoma cell lines, and that M2 macrophages induced more proliferation than did M1 macrophages. Stat3 activation in lymphoma cells was involved in this cell-cell interaction. Cytokine array analysis demonstrated that complement 5a (C5a) was detected in supernatants of M2 macrophages, but not in those of M1 macrophages or lymphoma cells. Although we demonstrated M2 macrophage-derived C5a is one of growth factors and a Stat3 activator, C5a was not a predominant molecule associated to lymphoma cell activation induced by M2 macrophages. However, these findings provide novel insights into the molecular mechanism related to M2 macrophages and lymphoma cell interaction. [*J Clin Exp Hematop* 53(2) : 127-133, 2013]

Keywords: C5a, cell-cell interaction, CD163, TAM, Stat3

INTRODUCTION

Macrophages that have infiltrated into tumor tissues are referred to as tumor-associated macrophages (TAMs) and are now of much interest because of their functions in the tumor microenvironment.¹⁻³ Recent studies revealed the possible involvement of TAMs, especially anti-inflammatory M2 TAMs, in several malignant tumors, including hematological malignancies.⁴ In Hodgkin lymphoma, diffuse large B-cell lymphoma, and angioimmunoblastic T-cell lymphoma, the number or ratio of CD163⁺ M2 TAMs is positively associated with worse clinical prognosis.⁵⁻⁸ In primary central nervous system lymphoma (PCNSL), higher Stat3 (signal transducer and activator of transcription 3) activation in lymphoma cells was

found to be closely associated with higher numbers of M2 TAMs; however, no correlation between M2 TAMs and clinical prognosis was seen.⁹

Stat3 is one of the important signal molecules related to angiogenesis, immunosuppression, cell survival, and cell proliferation, and higher Stat3 activation in tumor cells is significantly associated with lower survival rates of patients with several malignant tumor types.^{10,11} Our previous studies revealed that Stat3 activation in PCNSL,⁹ renal cell carcinoma,¹² ovarian cancer,¹³ and glioma cells¹⁴ was strongly induced by co-culture with M2 macrophages; however, detailed mechanisms regarding cell-cell interactions contributing to this Stat3 activation remain unclear. Therefore, this study was designed to investigate the significance of Stat3 activation for lymphoma cell proliferation using *in vitro* co-culture experiments.

MATERIALS AND METHODS

Macrophage culture

Peripheral blood mononuclear cells were isolated from 2 healthy volunteer donors, from whom written informed consent for experimental use was obtained. CD14⁺ monocytes were isolated using CD14-microbeads (Miltenyi Biotec, Bergisch

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Gladbach, Germany). Monocytes were plated in 24-well plates (6×10^4 cells/well) and cultured with granulocyte macrophage-colony stimulating factor (5 ng/mL) (WAKO, Tokyo, Japan) for 5 days to induce differentiation into immature macrophages, and cultured with macrophage-colony stimulating factor (M-CSF) (200 ng/mL) (WAKO) to induce differentiation to immature M2 macrophages, as described in previous reports.^{15,16} After gentle washing in phosphate-buffered saline, immature macrophages were stimulated with interferon- γ (2 ng/mL) (Pepro Tec, Rocky Hill, NJ, USA) to induce mature M1 macrophages and immature M2 macrophages were stimulated with interleukin-10 (10 ng/mL, Pepro Tec) to induce mature M2 macrophages.

Cell lines

B-cell lymphoma cell lines (SLVL, Raji and Daudi) were obtained from Cell Bank, RIKEN BioResource Center (Tsukuba, Japan) and were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum.⁹ The mycoplasma test was performed using a polymerase chain reaction detection kit (Takara Bio Inc, Otsu, Japan). In some experiments, SLVL cells (5,000 cells/well in 96-well plate) were cultured with human complement 5a (C5a) (R&D systems, Minneapolis, MN, USA) for 24 hrs to investigate its effects on cell proliferation. C5a receptor antagonist (Tetrahydronaphthalenyl carboxamide, Merck, Billerica, MA, USA) was used at concentration of 1 and 10 mM.

Co-culture experiments

Lymphoma cell lines (5×10^4 cells/well) and macrophages were co-cultured in Dulbecco's modified Eagle's medium supplemented with 2% fetal bovine serum for 3 days in 24-well plates. In indirect co-culture experiments, transwell chamber dishes (Roche, Mannheim, Germany) were used to separate the macrophages and lymphoma cells.

5-bromo-2'-deoxyuridine (BrdU) incorporation and immunostaining

BrdU incorporation and immunostaining were performed using the BrdU ELISA kit (Roche), according to the manufacturer's protocol, with minor modifications. Briefly, after culture with BrdU for 90 min, cells were attached to slide glasses by cytospin and fixed by acetone. CD204 (1 mg/mL, clone SRA-E5, Transgenic, Kumamoto, Japan) was stained as a pan-macrophage marker and visualized using a diaminobenzidine substrate system (Nichirei, Tokyo, Japan). After washes in glycine buffer (pH2.2), cells were stained by anti-BrdU antibody and visualized using the HistoGreen solution (Linaris Biologische, Wertheim-Bettingen, Germany).

Small interfering RNA (siRNA) in human macrophages

Lymphoma cells were transfected with siRNA against human Stat3 (Santa Cruz Biotech, Santa Cruz, CA, USA) using Lipofectamine RNAi MAX (Invitrogen, Carlsbad, CA, USA). Control siRNA (Santa Cruz Biotech) was used as a negative control. Down-regulation of Stat3 protein was confirmed by Western blot analysis.

Western blot analysis

Cellular proteins were solubilized in Tris buffer containing 2% SDS, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride and Phosphatase Inhibitor Cocktail (Nacalai Tesque, Tokyo, Japan). The amount of protein was quantified by the bicinchoninic acid assay; equal amounts of protein were separated on SDS-PAGE, and then were transferred to a polyvinylidene fluoride membrane. Following blocking in Tris buffer containing 2% bovine serum albumin, the membrane was stained with anti-Stat3 (0.2 mg/mL, F-2) and anti-pStat3 (0.2 mg/mL, pS727.49) antibodies (Santa Cruz Biotech), according to the manufacturer's protocol.

RT-PCR for C5a receptors

Total RNA was isolated from SLVL and Raji cells using RNAiso plus (Takara Bio Inc). RNA was reverse-transcribed by means of the PrimeScript RT reagent kit (Takara Bio Inc). The cDNA product was amplified by PCR. Primers were as follows: CD88, 5'-TGGGCACTATGATGACAACC-3' (forward) and 5'-CGACTGCAAAGATGACCAAG-3' (reverse); C5L2, 5'-CTGTATTTTGGGAGGGCTCA-3' (forward) and 5'-GGGCAGGATTTGTGTCTGTT-3' (reverse). The housekeeping gene GAPDH (glyceraldehyde 3-phosphate dehydrogenase) was used as the PCR control. PCR products were run on 2.0% agarose gels and stained with SYBR Safe DNA gel stain (Invitrogen).

Cytokine array

Cytokine array analysis was performed using a human cytokine array kit, panel A (R&D Systems), according to the manufacturer's protocol.

Statistical analysis

All *in vitro* data represent two or three independent experiments ($n = 3$ or 4 samples each) and are expressed as means \pm SD, and the Mann-Whitney U-test was used for two-group comparisons. A value of $p < 0.05$ was considered statistically significant. Statistical analysis was performed using StatMate III software (ATOMS, Tokyo, Japan).

RESULTS

Significant proliferation of lymphoma cells was induced by direct co-culture with M2 macrophages

Following co-culture of SLVL cells (a B-cell lymphoma cell line) with immature, M1, or M2 macrophages for 3 days, the BrdU incorporation assay and double-immunostaining was performed to evaluate lymphoma cell proliferation. As shown in Fig. 1A, BrdU incorporation into SLVL cells was

significantly induced by direct co-culture with M2 macrophages. The size of SLVL cell nuclei was also enlarged by co-culture with M2 macrophages (Fig. 1B). The longest length of nuclei in SLVL cells with or without co-culture was 18.8 ± 2.7 mm and 15.7 ± 2.8 mm respectively ($p = 0.003$, $n = 20$). When lymphoma cell proliferation in co-culture with the three macrophage types was compared, it was found to be elevated by all three (Fig. 1C), and to a notably greater extent by M2 macrophages. Similar results were obtained using other B-cell lymphoma cell lines, such as Daudi cells

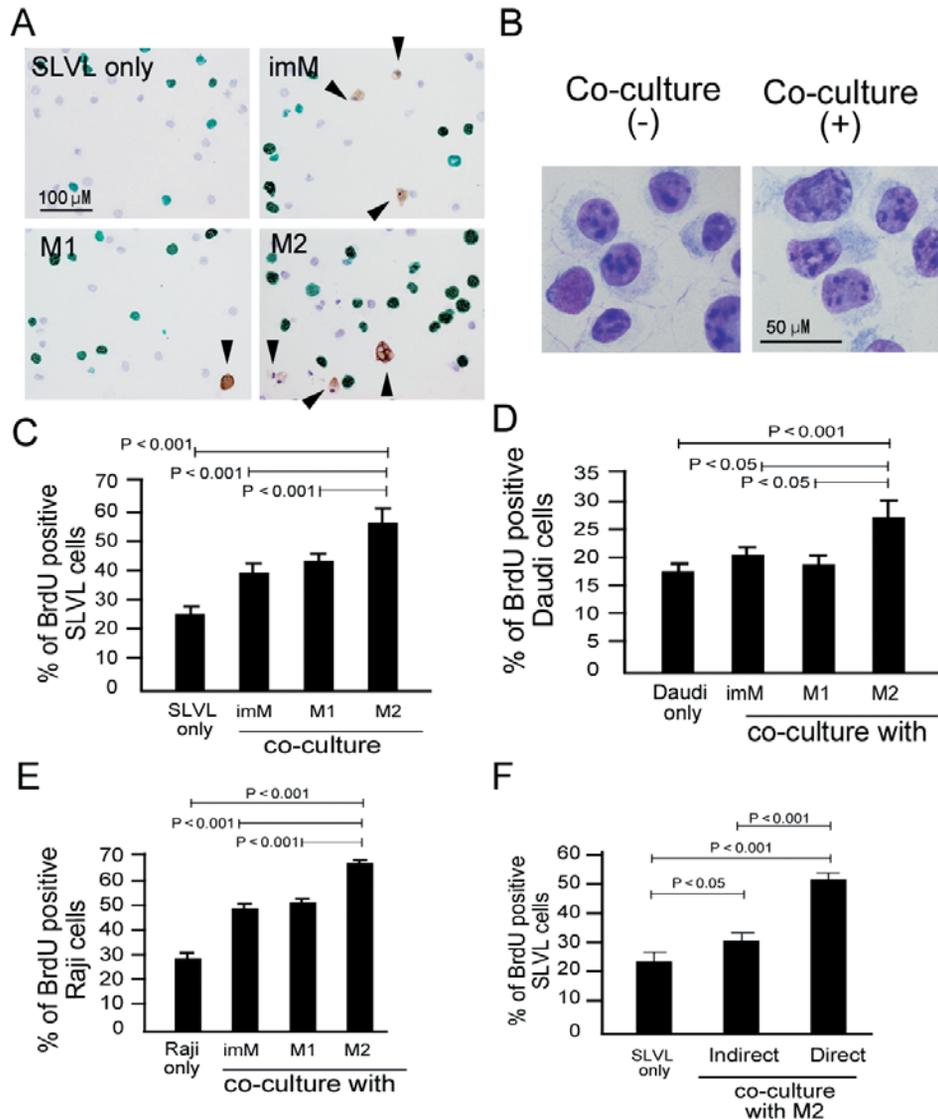


Fig. 1. Cell-cell interaction between lymphoma cells and macrophages. (1A) SLVL cells directly co-cultured with immature (*imM*), M1, or M2 macrophages, and then double immunostained [green, 5-bromo-2'-deoxyuridine (BrdU); brown, CD204 as a macrophage marker; blue, nuclear staining] to evaluate BrdU incorporation in CD204⁻ SLVL cells. Arrows mean CD204⁺ macrophages. (1B) SLVL cells, with or without co-culture, stained by a Diff-Quick solution. Percentage of BrdU⁺ (1C) SLVL, (1D) Daudi and (1E) Raji cells in co-culture experiments. (1F) BrdU incorporation in indirect and direct co-culture experiments.

(Fig. 1D) and Raji cells (Fig. 1E). In addition, higher BrdU incorporation in SLVL cells was induced by direct co-culture with M2 macrophages, as compared to indirect co-culture using the transwell culture system (Fig. 1F).

Lymphoma cell proliferation induced by co-culture with macrophages was dependent on Stat3 activation

To test the significance of Stat3 activation in cell-cell interactions between lymphoma cells and macrophages, Stat3 protein in SLVL cells was down-regulated by siRNA (Fig. 2A) before macrophage co-culture experiments. Stat3 down-regulation in co-cultured SLVL cells significantly suppressed BrdU incorporation (Fig. 2B & 2C).

C5a was involved in cell-cell interactions between M2 macrophages and lymphoma cells

Use of a cytokine array kit identified elevated C5a in M2 macrophage supernatants (Fig. 3A) and as this is considered to activate Stat3, it became a focus of this study. SLVL cells expressed C5a receptors (Fig. 3B) and significant Stat3 activation was induced by co-culture with C5a recombinant protein (Fig. 3C). Cell proliferation and BrdU incorporation were also significantly induced by co-culture with C5a in SLVL cells (Fig. 3D & 3E). No expression of C5a receptors was observed in Raji cells (Fig. 3B), and C5a did not up-regulate the cell proliferation of Raji cells (data not shown). Lymphoma cell activation induced by C5a was suppressed by Stat3 down-regulation (Fig. 3F). C5a receptor antagonist slightly lowered the BrdU incorporation in SLVL cells co-

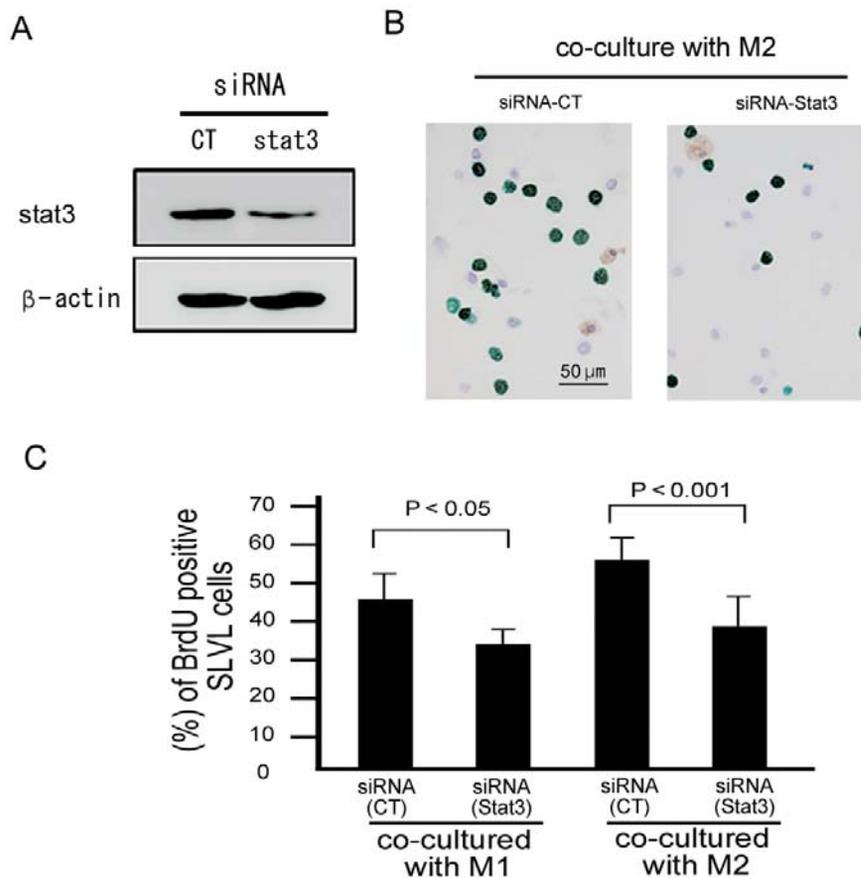


Fig. 2. Role of Stat3 in cell-cell interaction between lymphoma cells and macrophages. (2A) Western blot analysis confirmed suppression of Stat3 in SLVL cells. (2B) Stat3 down-regulated SLVL cells were co-cultured with M2 macrophages for 2 days, and double immunostained for 5-bromo-2'-deoxyuridine (BrdU) and CD204. (2C) In the same conditions, Stat3 down-regulated SLVL cells were co-cultured with M1 or M2 macrophages for 2 days, and after double immunostaining for BrdU and CD204, percentages of BrdU⁺ SLVL cells were calculated. siRNA, small interfering RNA

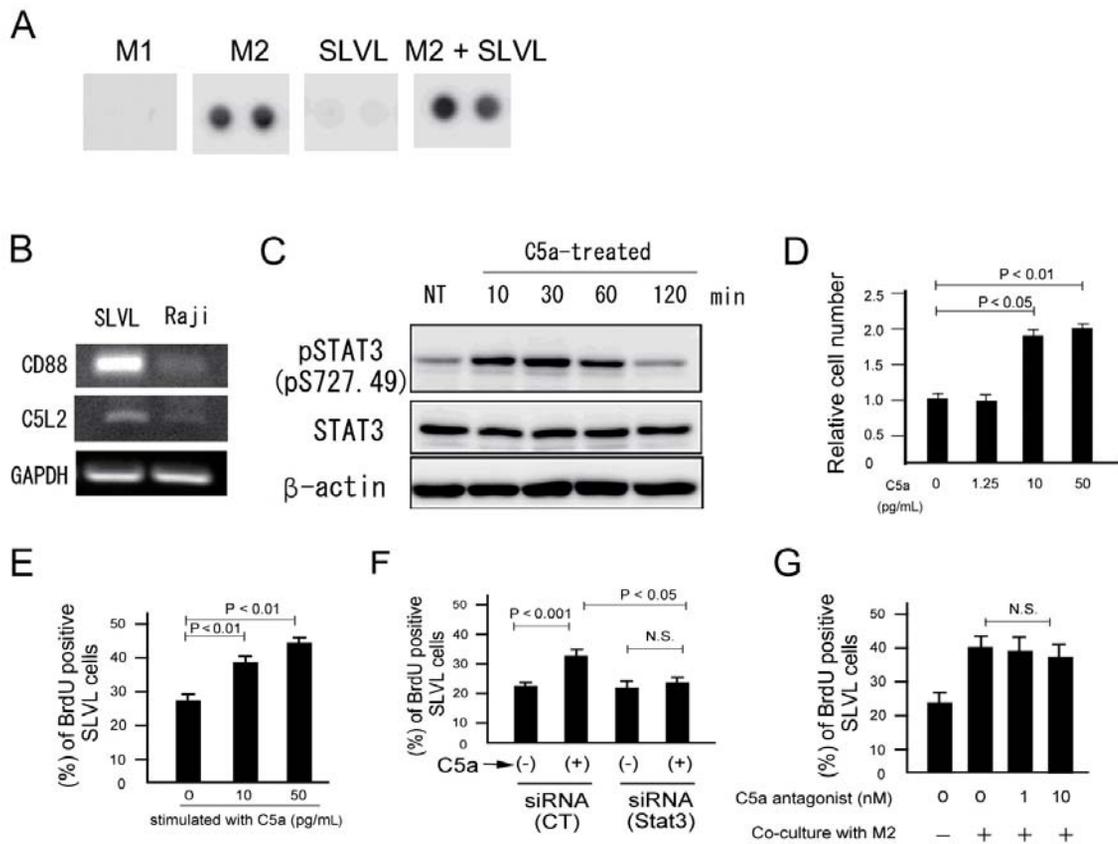


Fig. 3. M2 macrophage-derived C5a induced Stat3 activation and promoted SLVL cell proliferation. (3A) Monoculture (M1, M2 and SLVL) and co-culture (SLVL + M2) conditioned medium was prepared and soluble factors from each were detected by a human cytokine array kit. (3B) Expression of C5a receptors (CD88 and C5L2) was detected by RT-PCR. (3C) SLVL cells were incubated with 10 pg/mL human recombinant C5a for 10 to 120 min, and Stat3 activation was determined at 10, 30 and 60 min. (3D) SLVL cell proliferation by C5a stimulation was evaluated by WST-1 {4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolol]-1,3-benzene disulfonate} assay. (3E) SLVL cells were cultured with or without C5a and then 5-bromo-2'-deoxyuridine (BrdU) incorporation was evaluated. (3F) Following Stat3 protein was down-regulated by small interfering RNA (siRNA), SLVL cells were cultured with or without C5a and then BrdU incorporation was evaluated. (3G) SLVL cells and M2 macrophages were co-cultured with or without C5a antagonist and then BrdU incorporation was evaluated.

cultured with M2 macrophages, however, the data was statistically not significant (Fig. 3G).

DISCUSSION

Results of a previous study revealed that Stat3 activation in PCNSL tumor cells was closely associated with the number of M2 macrophages present in tumor samples, and that Stat3 in SLVL cells was activated by indirect co-culture with M2 macrophages.⁹ However, our group previously had not investigated the detail mechanisms of cell-cell interactions. In this study, we showed BrdU incorporation in SLVL cells was also strongly up-regulated by direct co-culture with M2 macrophages, compared to direct co-culture with M1 macrophages or indirect co-culture with M2 macrophages. This M2

macrophage-induced SLVL cell activation was mediated by Stat3 activation, and we suggested Stat3 activation was induced by soluble factors derived from activated macrophages. Our previous research using glioma cells reported that M-CSF expression on cell surface of tumor cells induced strong macrophage activation which in turn up-regulated the growth factor productions from macrophages,^{12,14} but M-CSF expression was not detected in lymphoma cell lines.

In this study, C5a was found to be secreted into M2 macrophage supernatants and to activate SLVL cells via Stat3 signaling, however C5a was not involved in the activation of Raji cells. This observation is consistent with our previous observation in which M2 macrophages induced Stat3 activation in SLVL cells but not in Raji cells.⁹ These data may indicate the engagement of macrophage-derived C5a is re-

stricted to lymphoma cells which express C5a receptors.

C5a is a known anaphylatoxin that activates the mitogen activated protein kinase and Stat3 signaling pathway.^{17,18} Proinflammatory factors secreted from macrophages, such as HMGB-1, are significantly elevated by C5a in an autocrine manner, and blocking C5a largely reverses the lethal inflammatory reaction in an animal model of septic shock.¹⁹ In this study, C5a receptor antagonist did not significantly suppress the BrdU incorporation in lymphoma cells co-cultured with M2 macrophages. This indicated that soluble factors other than C5a may also be involved in cell-cell interaction between lymphoma cells and M2 macrophages. Actually, in this study using cytokine array, some cytokines such as interleukin-6 and GRO- α which probably related to Stat3 signal were also secreted by M1 and M2 macrophages (unpublished data). The cytokine array kit used in the present study included 36 different molecules, however, many other molecules related to Stat3 activation, such as vascular endothelial growth factor, epidermal growth factor, and prostaglandin E2, are known to be secreted by macrophages.^{20,21} Further studies are necessary to elucidate the precise mechanisms underlying cell-cell interactions between macrophages and lymphoma cells.

Recent reports have implied that blocking Stat3 activation would be efficacious for patients with malignant tumors.²² Some Stat3 inhibitors have been studied in the past several years, and have significantly suppressed tumor progression or metastasis in murine cancer models.^{23,24} Since Stat3 activation is also involved in angiogenesis and immunosuppression, Stat3 inhibition is considered effective anti-cancer therapy through not only direct effects on the cancer cells but also by inhibitory effects on vessel formation and on immune escape in the tumor microenvironment.¹¹ In a murine B-cell lymphoma model, use of a Stat3 inhibitor increased anti-lymphoma immunity *in vivo*.²⁵

In conclusion, the present study indicates that Stat3 activation in lymphoma cells due to cell-cell interaction with M2 macrophages is important for the development of the tumor microenvironment. Stat3 activation by macrophage-derived C5a might be one of mechanisms of cell-cell interaction. In addition, Stat3 inhibitors or compounds which deactivate M2 macrophage could prove to be effective adjunctive therapeutic agents for malignant lymphoma patients.

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