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Spirulina Complex Polysaccharides Suppress the Growth of Glioma in T Lymphocyte- and Macrophage-dependent Manner

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ABSTRACT

Murine RSV-M glioma cells have a feature of endothelial cells. Indeed, they express an endothelial cell marker CD31 *in vitro*. However, CD31 expression was not observed in RSV-M cells growing *in vivo*. We have reported that down-regulating angiogenesis with *Spirulina* complex polysaccharides (CPS) suppressed glioma growth via a Toll-like receptor (TLR) 4 signal. RSV-M cells were implanted subcutaneously (S.C.) in C3H/HeN mice and into TLR4 mutant C3H/HeJ mice. Treatment with either *Spirulina* CPS or *Escherichia coli* (*E. coli)* lipopolysaccharides (LPS) strongly suppressed RSV-M glioma cell growth in C3H/HeN but not C3H/HeJ mice. Both *Spirulina* CPS and *E. coli* LPS induced NFκB activation and their responses were suppressed by pre-incubating these polysaccharides with polymyxin B, suggesting that the chemical nature is shared between these two complex polysaccharides. Administration of anti-CD8 antibodies, anti-CD4 plus anti-CD8 antibodies or anti-asialo GM1 antibodies enhanced glioma growth, suggesting that T cells, and natural killer (NK) cells or macrophages were involved in the suppression of the tumor growth by *Spirulina* CPS. In this review, we focused on examining whether macrophages or NK cells are responsible for the suppression of the glioma growth. We found that both *Spirulina* CPS and *E. coli* LPS suppressed tumor growth by activating F4/80 positive macrophages. In conclusion, *Spirulina* CPS suppressed the tumor growth by activating both T cells and macrophages and down-regulating angiogenesis. On the other hand, *E. coli* LPS suppressed glioma growth by activating macrophages or NK cells but not T cells, without changing the level of angiogenesis.

Key words : *Spirulina* CPS, NFκB, angiogenesis, TLR4

1. Introduction

We have explored the toll-like receptor (TLR) responsive materials from algae such as cyanobacterium, blue-green alga. We found that *Spirulina* CPS are effective in inducing NFκB activation in response to either TLR2 or TLR4 (Tominaga *et al*., 2010). *Spirulina* (*Arthrospira platensis*) is a Gram-negative, oxygenic, photosynthetic, and filamentous cyanobacterium that both the Aztecs living near Lake Texcoco in Mexico and the Kanembu tribe, residing near Lake Chad in Africa, consumed as a food. It is reported that the Kanembu tribe made dried biscuits called "dihe" from *Spirulina* collected from the waters of the alkaline lakes in the Kanem area, northeast of Lake Chad and ingested them historically as part of a daily protein diet (Belay *et al*., 2008). We used *Spirulina pacifica* that was selected from a strain of edible *Arthrospira platensis* in 1984 by Cyanotech (a generous gift from Dr. Gerald Cysewski, Cyabotech, Kailua-Kona, Hawaii). Westphal fractions (hot phenol extracts) of *Spirulina* were prepared as described by Westphal *et al*., 1965, and Kawanishi, *et al*., 2013 (a generous gift from Dr. Satoshi Fukuoka).

We have compared TLR2 or TLR4 responsive materials among algae extracts using Golenbock's Chinese hamster ovary derived fibroblast like cells (CHO cells) engineered to express human CD25 in response to NFκB induced by the TLR2- or TLR4-binding molecules

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(Delude *et al*., 1998, Medvedev *et al*., 2001). Alginic acid prepared from *Macrocystis pyrifera* (Kelp) stimulated TLR4 but not TLR2 to activate NFκB. Fucoidan from *Fucus vesiculosus* activated NFκB efficiently via TLR4 but weakly via TLR2 signaling. Polysaccharides from *Petalonia binghamiae* activated NFκB weakly in TLR2- and TLR4-dependent manner. Westphal fraction of *Escherichia coli*, lipopolysaccharides (LPS) stimulated TLR4 much more efficiently to activate NFκB compared to TLR2. In contrast, the Westphal fraction of *Spirulina pacifica* (*Spirulina* complex polysaccharides: *Spirulina* CPS) activated NFκB weakly via either the TLR2 or TLR4 pathway (Tominaga *et al*., 2010).

Although *E. coli* LPS suppressed the growth of gliomas inoculated subcutaneously in a TLR4-dependent manner, it is well known that *E. coli* LPS are highly toxic (Chicoine *et al*., 2007). Therefore, we compared the anti-tumor activity of *Spirulina* CPS with that of *E. coli* LPS against gliomas (Kawanishi *et al*., 2013) and found that both *Spirulina* CPS and *E. coli* LPS strongly suppressed RSV-M glioma cell growth in C3H/HeN, but not C3H/HeJ, mice. By the re-challenge experiments, we noticed that *Spirulina* CPS but not *E. coli* LPS enhance the ability to generate acquired immunity. We proved that the anti-glioma activity of *Spirulina* CPS depends on $CD8^+$ and $CD4^+$ T cells through depletion experiments using anti-CD4 or anti-CD8 antibodies (Kawanishi *et al*., 2013). We also showed suggestive evidence that *Spirulina* CPS have a unique feature in that they suppress angiogenesis, in part by regulating the serum level of IL-17 (Kawanishi *et al*., 2013).

In this review, we focused on macrophages that are activated in gliomas when *Spirulina* CPS are administered. We found that *Spirulina* CPS activated both T cells and macrophages in gliomas, an effect which is negatively correlated with the level of angiogenesis in gliomas. We also discuss the nature of gliomas in terms of expression of CD31.

2. Similarity of chemical natures between *E. coli* LPS and *Spirulina* CPS

Although *E. coli* LPS induced a higher level of NFκB than *Spirulina* CPS as measured in the expression intensity of human CD25 in Chinese hamster ovary cells 3E10 ($TLR2^-$ and $TLR4^+$) (Tominaga *et al*., 2010), *Spirulina* CPS induced a weak but significant level of NFκB at a concentration of one microgram/ml (Fig. 1). We pre-incubated both *E. coli* LPS and *Spirulina* CPS with polymyxin B to see the effects of this antibiotic on the abilities of these polysaccharides to induce the expression of NFκB in 3E10 cells. As shown in Fig. 1,

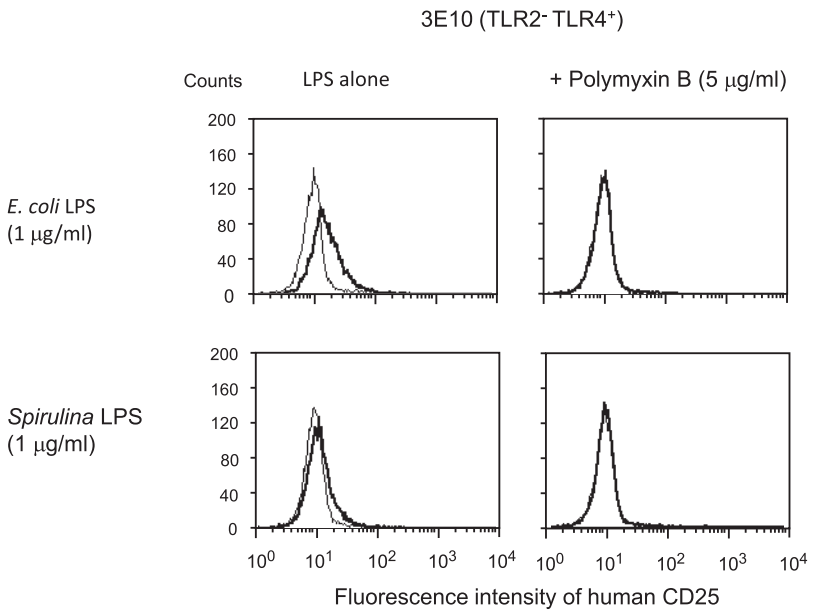

Fig. 1. Polymyxin B suppresses NFκB-inducing ability of *E. coli* LPS and *Spirulina* CPS.
NFκB generating ability of each polysaccharide fraction was detected by measuring the expression level of human CD25 in 3E10 Chinese hamster ovary cells established by Golenbock. Human CD25 was detected by phycoerythrin-coupled anti-human CD25 antibodies and the fluorescence intensity was detected by a flowcytometer. The background is shown as the left-hand peak expressed with a thinner line in each panel that is stained with phycoerythrin-coupled control IgG. Levels of human CD25 induced by both polysaccharides were expressed in bold lines. In right panels, both *E. coli* LPS and *Spirulina* CPS (100 μg/ml) were pre-incubated with polymyxin B (500 μg/ml) at room temperature for 60 minutes before stimulating 3E10 cells. Polymyxin B abolished the expression of human CD25 on 3E10 cells induced by either *E. coli* LPS or *Spirulina* CPS.

polymyxin B could abolish the NFκB inducing activity of both polysaccharides, suggesting that they share the same chemical nature. It is highly possible that *Spirulina* CPS have a moiety that resembles lipid A, since polymyxin B reportedly binds to the lipid A portion of bacterial LPS (Morrison *et al*., 1976).

3. Anti-tumor activities of *Spirulina* CPS against murine RSV-M glioma depend on the TLR4 pathway

In order to examine whether anti-tumor activity of *Spirulina* CPS depends on TLR4, we administered *Spirulina* CPS intraperitoneally to RSV-M glioma-bearing C3H/HeN and TLR4-mutant C3H/HeJ mice weekly starting six days after inoculating glioma tumor cells subcutaneously on the back of these mice. Both *Spirulina* CPS and *E. coli* LPS significantly suppressed the growth of the glioma in C3H/HeN but not C3H/HeJ mice (Fig. 2). This data clearly suggests that *Spirulina* CPS suppress the growth of RSV-M glioma by stimulating the pathway through TLR4 as *E. coli* LPS does. We will describe the difference between *E. coli* LPS and *Spirulina* CPS in terms of the regulation of immunity against RSV-M glioma cells through activities such as cytokine production and the use of effector cells against glioma cells.

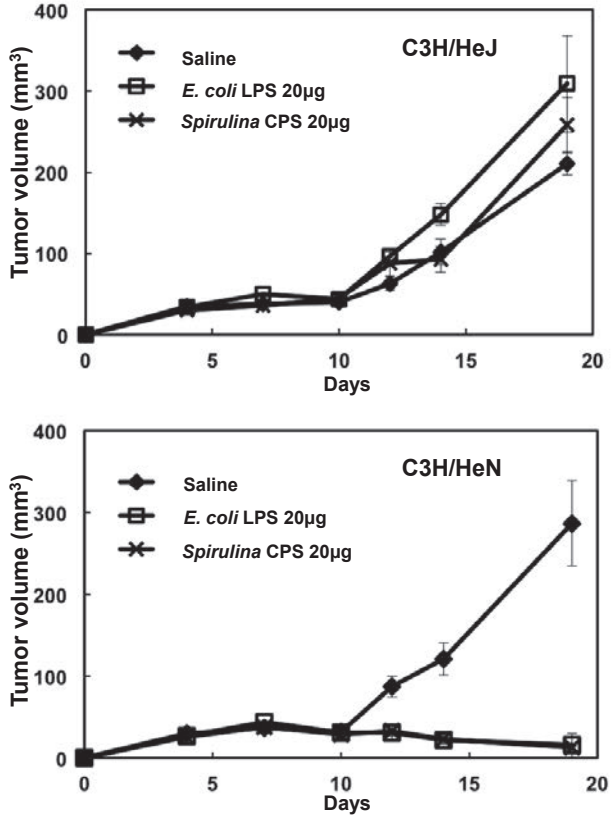

Fig. 2. Effects of *E. coli* LPS and *Spirulina* CPS on RSV-M glioma cell growth in C3H/HeJ and C3H/HeN.
Five million RSV-M glioma cells were inoculated subcutaneously on the backs of C3H/HeN or C3H/HeJ mice. *Spirulina* CPS (20 μg), *E. coli* LPS (20 μg), or saline were injected intraperitoneally every week starting six days after tumor inoculation. Tumor volume was calculated using the formula, volume = width^2 x length/2 and the data were represented as average ± standard error (SEM) of 6 female 10-week-old mice per group. There are significant differences between the following groups ($P < 0.01$; Student's *t*-test): Saline-treated group versus *Spirulina* CPS-treated group and Saline-treated group versus *E. coli* LPS-treated group on days 12, 14, and 19 after tumor transplantation in C3H/HeN mice. From Kawanishi *et al*., 2013.

4. IL-17 is induced by the inoculation of glioma cells, and *Spirulina* CPS suppressed the serum level of IL-17 of glioma bearing mice

We observed that anti-IFN-γ antibodies did not have any effects when administered four days after inoculation of RSV-M glioma cells in either C3H/HeJ or C3H/HeN mice. In contrast, anti-IL-17 antibodies significantly suppressed the growth of glioma in both strains of mice (Kawanishi *et al*., 2013). Furthermore, we did not observe any additive effect in terms of suppression of RSV-M glioma cells between anti-IL-17 antibodies and *Spirulina* CPS in C3H/HeN mice. This result suggests that *Spirulina* CPS may suppress the growth of RSV-M glioma cells by down-regulating the production of IL-17.

We measured the level of IL-17 in the serum of tumor bearing mice 13 days after tumor inoculation (Fig. 3). Inoculation of RSV-M glioma cells increased the serum level of IL-17 in both C3H/HeJ and C3H/HeN mice. *E. coli* LPS increased the serum level of IL-17 in glioma-bearing C3H/HeN but not C3H/HeJ mice. In contrast, *Spirulina* CPS decreased the serum level of IL-17 in glioma-bearing C3H/HeN but not C3H/HeJ mice. Numasaki *et al*. reported that IL-17 promotes angiogenesis not only via stimulation of vascular endothelial cell migration but also via elaboration of proangiogenic factors, which creates an imbalance between angiogenesis activators and inhibitors in the vasculature (Numasaki *et al*., 2003). We reported that there were fewer endothelial cells expressing CD31 in RSV-M gliomas from *Spirulina* CPS-treated mice than in those from control mice or *E. coli* LPS-treated mice (Kawanishi *et al*., 2013).

5. T lymphocytes and macrophages are responsible for the suppression of growth of RSV-M glioma by *Spirulina* complex polysaccharides

To identify the effector cells that suppress the

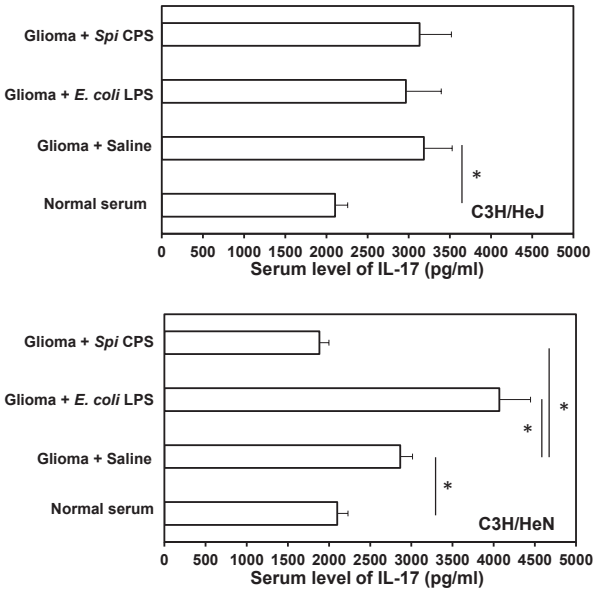

Fig. 3. Serum concentrations of IL-17 in glioma RSV-M bearing mice treated with Spirulina CPS.
On day 13 after tumor inoculation in the experiment of Fig. 2, serum from individual mouse was prepared and IL-17 levels were measured using an ELISA as described (Kawanishi *et al*., 2013). *Spi* CPS: *Spirulina pacifica* complex polysaccharides. Results are shown as mean values of 6 mice (pg/ml ± SEM). *: $P < 0.01$, between groups paired with lines (Student's *t*-test). Modified from reference by Kawanishi *et al*., 2013.

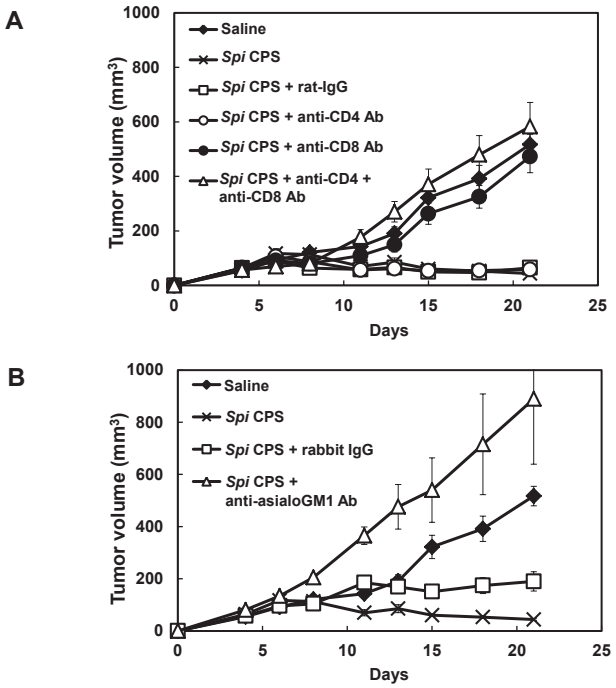

Fig. 4. Both T cells and asialo GM1 positive cells are involved in glioma-suppressing activity of *Spirulina* CPS.
Five million RSV-M glioma cells were inoculated subcutaneously on the back of C3H/HeN mice. *Spirulina* CPS (100 μg) or saline was injected intraperitoneally every week starting six days after tumor inoculation. Data represent means ± SEM of 5 female 10-week-old mice per group. Antibodies were administered as described (Kawanishi *et al*., 2013). In brief, antibodies were administered at a dose of 100 μg/100 μl/mouse on days -1, 0, and +3 before and after tumor transplantation. **(A)** Effects of anti-CD4 and anti-CD8 antibodies on the suppressive effect of *Spirulina* CPS (*Spi* CPS) on the growth of glioma cells in C3H/HeN mice. There is a significant difference between the following groups at the indicated periods ($P<0.05$; student's *t*-test): Saline versus *Spi* CPS; *Spi* CPS + anti-CD8 antibodies versus *Spi* CPS + rat IgG from days 13 to 21; *Spi* CPS + anti-CD8 antibodies versus *Spi* CPS + anti-CD4 + anti-CD8 antibodies from days 13 to 18. **(B)** Effect of anti-asialo GM1 antibodies on the suppressive effect of *Spirulina* CPS on the growth of glioma cells in C3H/HeN mice. There is a significant difference between the following groups at the indicated periods ($P<0.05$; student's *t*-test): *Spi* CPS + anti-asialo GM1 antibodies versus *Spi* CPS + rabbit IgG from days 11 to 21. From Kawanishi *et al*., 2013.

growth of RSV-M glioma cells when *Spirulina* CPS are administered, we depleted the subpopulation of cells that may be engaged in this regulation by administering antibodies against the surface marker of each subpopulation. As we reported, we found that anti-CD8 antibodies enhanced glioma growth when glioma-bearing mice were treated with *Spirulina* CPS, suggesting that $CD8^+$ T cells are involved in suppression of tumor growth by *Spirulina* CPS. Although anti-CD4 antibodies alone had no effect on tumor growth, they enhanced the glioma growth when administered together with anti-CD8 antibodies. These results suggest that $CD4^+$ T cells are also involved in tumor immunity induced by *Spirulina* CPS against this glioma. Anti-asialo GM1 antibodies enhanced tumor growth as efficiently as anti-CD4 and anti-CD8 antibodies, suggesting that NK cells or macrophages are involved in the suppression of the growth of RSV-M glioma cells by *Spirulina* CPS.

We tried to identify the asialo GM1-positive effector cells that are engaged in the suppression of the growth of RSV-M glioma cells. We observed no difference in the number of NK cells stained with anti-CD49b antibodies (clone DX5) between *Spirulina* CPS-treated and control glioma-bearing mice (data not shown). By staining F4/80 antigens in the section of gliomas growing in C3H/HeN mice, we found that the number of F4/80 positive macrophages migrated into the glioma by the treatment with *Spirulina* CPS are decreased after the treatment with either anti-CD8 antibodies, anti-CD4 antibodies plus anti-CD8 antibodies, or anti-asialo GM1 antibodies. *Spirulina* CPS induced more F4/80 macrophage migration into the tumor mass than *E. coli* LPS (Fig. 5A, B).

Then, we examined the CD31 expression in glioma cells in the same groups in which F4/80 positive cells were detected. Although both *E. coli* LPS and *Spirulina* CPS promoted the migration of F4/80 macrophages

into the glioma mass, only *Spirulina* CPS decreased the number of CD31-expressing cells. *E. coli* LPS had no effect on the number of CD31-expressing cells in the tumor mass (Fig. 5C). Since CD31 is a marker of endothelial cells, this result suggests that *Spirulina* CPS but not *E. coli* LPS suppressed the angiogenesis in the tumor of these RSV-M glioma cells. Interestingly, numbers of $CD31^+$ cells in glioma from each *Spirulina* CPS-treated mouse negatively correlated with the F4/80 positive macrophages in the glioma mass (Fig. 5, Fig. 6). The administration of anti-CD8 antibodies, anti-CD4 antibodies plus anti-CD8 antibodies, or anti-asialo GM1 antibodies in *Spirulina* CPS-treated mice decreased F4/80 positive macrophages and increased the CD31 positive endothelial cells in the glioma mass. In other words, the level of immune responses against RSV-M glioma cells are negatively correlated closely with the level of angiogenesis in gliomas of *Spirulina* CPS-treated mice. It is also suggested that T lymphocytes induced by *Spirulina* CPS are involved in the suppression of angiogenesis.

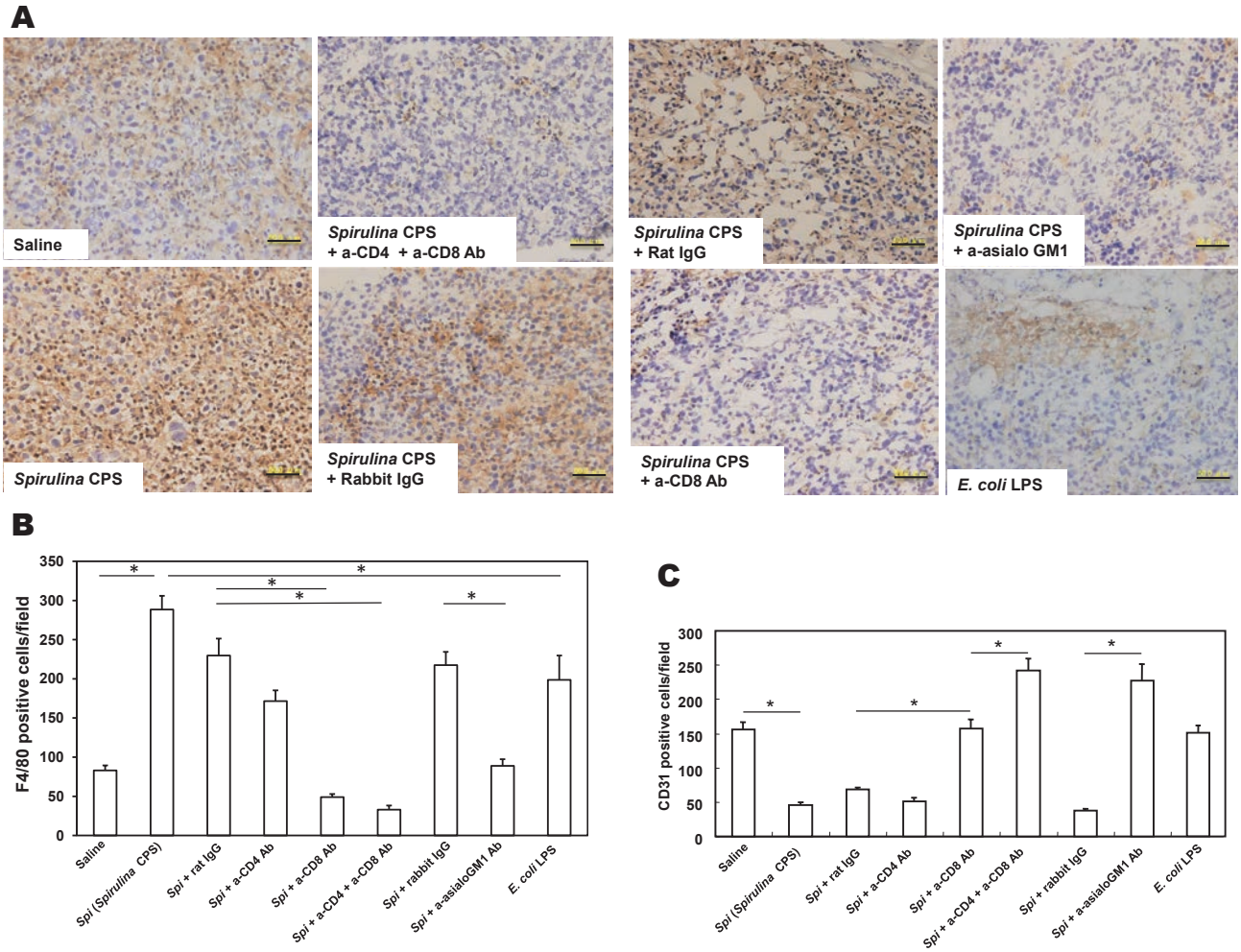

Fig. 5. Macrophages are involved in the glioma-suppressing activity of *Spirulina* CPS.
(A) Frozen sections of gliomas prepared from tumor-bearing C3H/HeN mice in **Fig. 4** were stained with anti-F4/80 antibodies (2.5 μg/ml, rat IgG2A, κ, clone: BM8, Bio Legend, San Diego CA) as described (Kawanishi *et al*., 2013). Representative photomicrographs are shown from experiments conducted in tumor samples from each group of mice. Bar, 50 μm. **(B)** F4/80 positive cells were compared among groups treated with anti-CD4 antibodies, anti-CD8 antibodies, anti-CD4 and anti-CD8 antibodies, and anti-asialo GM1 antibodies. F4/80 positive cells were counted in a field of 300 μm X 400 μm. Each group consisted of 10 fields randomly chosen from tumor sections of each treatment and cell numbers stained with anti-F4/80 antibodies are shown as mean values ± SEM. Asterisks indicate significant differences between the groups (P <0.01; ANOVA, Tukey-Kramer's post hoc test). **(C)** Frozen sections of gliomas prepared from tumor-bearing C3H/HeN mice in **(A)** were stained with anti-CD31 antibodies as described (Kawanishi *et al*., 2013). CD31 positive cells were compared among groups treated with anti-CD4 antibodies, anti-CD8 antibodies, anti-CD4 and anti-CD8 antibodies, and anti-asialo GM1 antibodies. CD31 positive cells were counted in a field of 300 μm X 400 μm. Each group consisted of 10 fields randomly chosen from tumor sections of each treatment and cell numbers stained with anti-CD31 antibodies are shown as mean values ± SEM. Asterisks indicate significant differences between the groups (P <0.01; ANOVA, Tukey-Kramer's post hoc test). (C) is modified from Kawanishi *et al*., 2013.

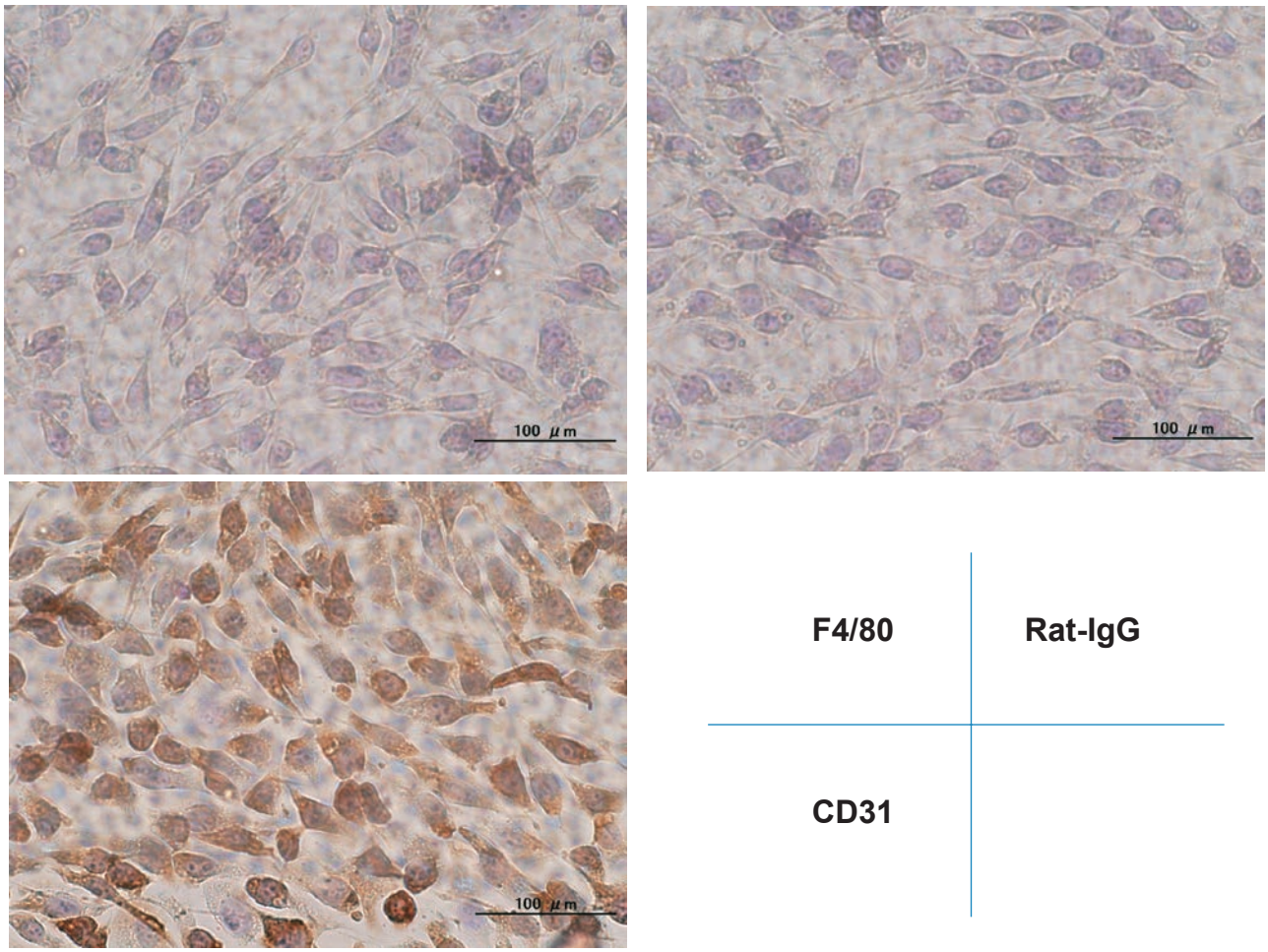

Fig. 6. Staining of RSV-M glioma cells with anti-CD31 antibodies *in vitro*.
In vitro RSV-M glioma cells were grown in Dulbecco's-modified Eagle medium with high glucose supplemented with 8% fetal calf serum, 20 U/ml penicillin, 50 μg/ml kanamycin. RSV-M glioma cells were stained with anti-CD31 antibodies (Rat IgG2a, κ, 390, BioLegend, San Siego, CA) using Simple stain mouse MAX-PO ($F(ab)'_2$ goat anti-Rat Ig and peroxidase coupled to the amino acid polymer) and 3,3'-diaminobenzidine according to the manufacture's protocol (Nichirei-Biosciences Inc., Tokyo, Japan) as described (Kawanishi *et al*., 2013). Samples were counterstained with hematoxylin.

6. RSV-M glioma cells express a higher level of CD31 *in vitro* compared with that *in vivo*

Ricci-Vitiani *et al*. reported that a variable number (range 20-90%, mean 60.7%) of endothelial cells in glioblastoma carry the same genomic alteration as tumor cells, indicating that a significant portion of the vascular endothelium has a neoplastic origin (Ricci-Vitiani *et al*., 2010). Wang *et al*. reported that a subpopulation of endothelial cells within glioblastomas harbor the same somatic mutations identified within tumor cells, such as amplification of EGFR and chromosome 7. They also demonstrated the stem-cell-like $CD133^+$ fraction includes a subset of vascular endothelial-cadherin (CD144)-expressing cells that show characteristics of endothelial progenitors capable of maturation into endothelial cells. They further showed that a subpopulation of the $CD133^+$ stem-like cell fraction is multipotent and capable of differentiation along tumor and endothelial lineages, possibly via an intermediate $CD133^+/CD144^+$ progenitor cell (Wang *et al*., 2010).

The process of neurogenesis in the adult brain has been primarily confined to the forebrain subventricular zone and subgranular zone of the dentate gyrus (Chesler *et al*., 2013). Within the subventricular zone of the mammalian brain, clusters of capillaries, associated growth factors such as brain-derived neurotrophic factor (BDNF), vascular endothelial growth factor (VEGF), and extracellular matrix components such as tenascin-C and chondroitin sulfate, create an environment which fosters stem cell growth and regulates proliferation and cell fate through cross talk and maintenance of local environmental factors such as pH and oxygen tension. Gliomas are typically highly vascularized tumors often with

extensive capillary beds that can provide a similar vascular niche for glioma initiating cells to reside in (Chesler *et al*., 2013). Calabrese *et al*. found that CD133+/Nestin$^+$ glioma-derived stem cells were closely associated with capillary associated endothelial cells (Calabrese *et al*., 2007). Golebiewska *et al*. reported that CD133-positive cells often associated with cancer stem cells in glioblastoma biopsies, do not represent a homogeneous cell population and include CD31-positive endothelial cells (Golebiewska *et al*., 2013).

We found that almost all RSV-M glioma cells express a high level of CD31 *in vitro* and this high level of expression of CD31 was not observed *in vivo* tumor (Fig. 6, Kawanishi *et al*., 2013). Once RSV-M glioma cells were inoculated subcutaneously on the back of mice, they decreased the expression level of CD31 and gradually formed the blood vessels to support their own growth.

We have hypothesized that the way *Spirulina* CPS inhibit angiogenesis is by down-regulating the production of IL-17. It is reported that TGF-β has been expressed in the normal subventricular zone, having a role in progenitor differentiation (Schneider *et al*., 1992). It is also found that glioblastoma or macrophages in glioma expressed TGF-β (Schneider *et al*., 1992). We have evidence that *Spirulina* CPS suppress the production of TGF-β and IL-6 by macrophages (unpublished observation). This result suggests that *Spirulina* CPS down-regulate the production of IL-17 by suppressing the production of TGF-β, IL-6, or IL-23 from macrophages. As described in Fig. 7, in the environment surrounding glioma cells, macrophages or other immune competent cells, or glioma cells by themselves secrete TGF-β, IL-6, or IL-23 that induce IL-17 producing cells, resulting in the high level of angiogenesis. Although *Spirulina* CPS reduce the level of IL-17 in the microenvironment surrounding glioma cells, *E. coli* LPS induce the production of TGF-β, IL-6, or IL-23 (unpublished observation) resulting in the high level of IL-17.

In conclusion, *Spirulina* CPS suppress the growth of glioma in a TLR4 dependent way by reducing the angiogenesis via down-regulation of the production of IL-17. Invasion of macrophages, CD4$^+$ T cells, and CD8$^+$ T cells are important for this suppression of angiogenesis. Since glioma cells are derived from stem cells that have characteristics of endothelial cells in the presence of TGF-β, *Spirulina* CPS may be an efficient reagent to suppress the growth of this tumor. As shown in Fig. 7, TGF-β also induces Treg cells that suppress the immunity against tumor cells. *Spirulina* CPS may also be useful to inhibit the suppression of antitumor immunity by Treg cells, because they have the ability to down-regulate the production of TGF-β.

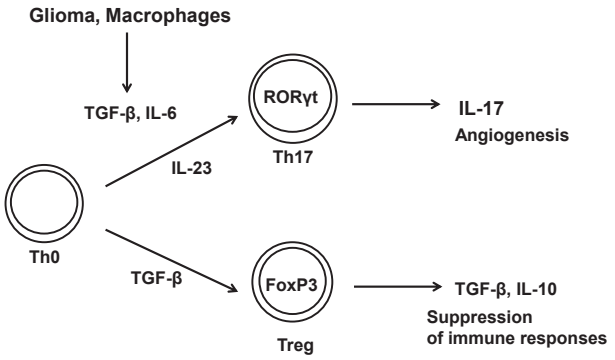

Fig. 7. Cytokines produced by glioma cells and the role of cytokines in the development of IL-17 producing cells.
A differentiation pathway of Th17 cells is illustrated in relation to that of Treg cells. Cytokines necessary for these differentiation pathways are described.

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