Introduction

The immune system has innate and acquired arms to eliminate foreign cells from the host. Innate system recognizes pathogenic microbes which possess pattern molecules serving as ligands for pattern-recognition receptors (1). These receptors reside in immune competent cells and trigger signaling to activate transcription factors in these cells (2). Many cytokines and cellular effectors are consequently induced to orchestrate host defense. We have a variety of foods that may contain many kinds of microbial patterns. Since intestinal and colon epithelial cells express pattern-recognition receptors (3,4), mucosal immune responses may be modulated by orally uptake of microbial material.

The cyanobacterium, Spirulina platensis, has been taken as a supplemental food for >15 years without any undesirable side-effect (5). Its safety for human consumption has been confirmed through toxicological studies (5). Spirulina contains a lipopolysaccharide (LPS)-like constituent that somewhat structurally differs from the bacterial LPS (6). Spirulina also contains the high content of protein, vitamins (especially A and B12) and minerals. It is rich in phenolic acids, tocopherols and γ-linolenic acid (5,7). Since Spirulina lacks cell walls, it is easily digested (7). Spirulina contains unique proteins, sugars and lipids (5,8) and these moieties of Spirulina are reported to participate in raising host immune responses including enhancement of Ab production (9), cytokine liberation (10), T cell response and NK activation (11). However, the exact molecular base responsible for these immune responses has not been clearly identified yet.

We previously reported that hot water-extract of Spirulina when orally taken in adult human enhances NK activation (11). This Spirulina activity is unique since most of the reported bacterial adjuvants facilitate CTL induction but not NK activation (12). For example, BCG-cell-wall skeleton (CWS) subcutaneously injected induces IL-12, IFN-γ and local skin erosion but no NK activation (13). TLR2 and TLR4 on myeloid dendritic cells (DCs) sense the peptidoglycan (PGN) of BCG (BCG-PGN) and induce DC maturation via the MyD88 pathway (13,14). BCG-PGN drives DCs to elicit a CTL-inducing state (15).

Here we show that hot water-extract of Spirulina activates NK in mice

even by oral administration. Having Spirulina confer both IFN-γ production and NK-mediated Rae-1-positive cell killing activity on mice. Consequently, Spirulina administration leads to retardation of implant tumor growth in mice. This Spirulina antitumor activity depends on MyD88 but not the other adaptor TICAM-1 (TRIF). Thus, Spirulina evokes a unique MyD88-dependent NK activation through mucosal immune responses. We offer the possible immune therapy in combination with BCG-CWS and Spirulina in this communication.

Materials and Methods

Reagents and antibodies

The following materials were obtained as indicated: Fetal calf serum (FCS) from Bio Whittaker (Walkersville, MD), mouse granulocyte-macrophage colony-stimulating factor (GM-CSF) and mouse IL-2 (mIL-2) from PeproTech EC, Ltd (London, UK), polymyxin B and lipopolysaccharide (LPS) (Escherichia coli O111:B4) from SIGMA-Aldrich (St. Louis, MO), synthetic macrophage-activating lipopeptide 2 (MALP-2) from Amersham Pharmacia Biotech (Piscataway, NJ) and Lympholyte-M from Cedarlane (Ontario, Canada). The enzyme-linked immunosorbent assay (ELISA) kits for mouse (m)IFN-γ from Amersham Biosciences.

The following antibodies were used: anti-mouse NKG2D polyclonal Ab and anti-pan-Rae1 polyclonal Ab (rabbit serum) were established in our laboratory (16), anti-asialoGM1 Ab from Seikagaku Kogyo Co., Ltd. or Wako Pure Chemical Industrials, Ltd (Tokyo, Japan). Monoclonal Abs against mouse CD4 and CD8β were kindly provided by Drs. T. Takahashi and K. Tsujimura (Aichi Cancer Center, Nagoya) as previously described (17). Fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit and rat IgG $F(ab')_2$ from American Qualex (San Clemente, CA), rat IgG1κ control FITC from BD PharMingen (San Diego, CA), and hamster IgG isotype control FITC from eBioscience (San Diego, CA).

Preparation of BCG-CWS and hot water-extract of Spirulina

Spray-dried powder of Spirulina platensis propagated under basic conditions (pH 11) in outdoor open tanks was extracted with water in an

autoclave for 1 h at 120 °C. In some experiments, citric acid was added to the hot water extract to adjust the pH to 4.0 (18). The water-soluble extract was prepared by removal of insoluble fractions by centrifugation. The Spirulina extract was adsorbed in the mouse food at 1 mg/g (Clea Japan, Tokyo). In other experiments to administer accurate amounts of the extract to mice, the soluble extract of Spirulina was condensed to 1 mg/ml for oral administration as described previously (18). In in vitro experiments, Spirulina extract was treated with polymyxin B (final concentration, 5 μg/ml) for 1 h at 37 °C before cell stimulation to exclude the effect of possible contaminating LPS.

BCG-CWS was prepared in Dr. I. Azuma's laboratory (Research Institute for Immunology, Hokkaido University) as described previously (19). The lot used in this study (Lot 10-2) consisted of mycolic acid, arabinogalactan and peptidoglycan with >97% purity and less than the detection limit of LPS contamination (data not shown). Since BCG-CWS is insoluble in water and organic solvents, the oil-in-water emulsion form of BCG-PGN micelles (BCG-emulsion) was used throughout the in vivo study. B16 cell debris conjugated to BCG-CWS (BCG-CWS/Ag) was prepared as described below and detailed in a report (15).

Mouse and cell lines

TLR2 -/-, TLR4 -/-, and MyD88 -/- mice were gifts from Dr. S. Akira (Osaka Univ., Osaka) as previously reported (15). TICAM-1 (TRIF) -/- mice were established in our laboratory (17). Female C57BL/6 mice were purchased from Clea Japan (Tokyo). Mice were maintained in our institute under specific pathogen-free conditions. All animal work was performed under guidelines established by the Osaka Medical Center Institutional Animal Care and Use Committee. Mice (12 weeks female C57BL/6) were housed four per cage and allowed food and water ad libitum. Animal studies were carefully performed without ethical problems. In some experiments, mice were fed for 3 weeks with pathogen-free food containing 1 mg/g Spirulina extract and control food with no Spirulina, which were prepared in Clea Japan. For more precise studies 600 μg/head of the Spirulina extract was directly administered to the mouse stomach via an injector.

B16D8 cell line was established in our laboratory as a subline of the B16 melanoma cell line (20). This subline was characterized by its low MHC levels with no metastatic properties when injected s.c. into syngeneic C57BL/6 mice (16,21). These cell lines were cultured in RPMI 1640/10% FCS. Tumor challenge studies were performed as previously described (15).

Preparation of BMDCs and spleen NK cells of mice

Mouse bone marrow-derived DCs (BMDCs) were prepared as described previously (15). Spleen NK cells were prepared by a reported method (22) with minor modifications (23). In brief, spleen cells were passed through a nylon wool column to remove B cells. In some cases, anti-asialoGM-1 Ab (100 μg) was injected intravenously into mice to eliminate NK cells (17). The nylon wool-nonadherent cells were incubated in tissue culture plates for 1 h to remove adherent cells. Nonadherent cells were subjected to the MACS system for the preparation of NK cells (23). The population was used as NK cells within 3 days.

Immunization

On day –28, -21, -14, -1 and +7 relative to the day of B16D8 challenge (Fig. 1), 5 x 10^6 B16D8 cells (in 90 μl) were in freeze-thaw cycles three times in PBS to prepare "debris" and the debris was mixed with 10 μl of 1 mg/ml BCG-CWS in emulsion buffer (BCG-CWS/Ag) (15). Wild-type mice (>12 weeks old) were s.c. immunized with 30 μl of this mixture per head at the base of the tail. The administration protocol is shown in a previous report (15). As controls, tumor debris (Ag) only or emulsion only was used. Since BCG-CWS alone in emulsion buffer possesses immune potentiation activity upon subcutaneous (s.c.) administration (15,18), it could not be employed as control for BCG-CWS/Ag.

Tumor challenge

B16D8 cells (2 x 10^6 cells in Fig. 1) were subcutaneously (s.c.) inoculated in the hind flank of wild-type mice. One group of the mice was i.v. injected with anti-asialoGM-1 Ab (100 μg/100 μ) every week and the other was with mouse IgG. The tumor-challenged mice were fed with

Spirulina-containing food from day 0 to the end of the study. In the next experiments, B16D8 cells (5 x 10^5 cells) were subcutaneously inoculated into mice, either preimmunized or non-immunized with BCG-CWS/Ag. Mice were grouped into four, each consisting of 20 mice. Group 1 with BCG-CWS/Ag and Spirulina, group 2 with BCG-CWS/Ag only, group 3 with Spirulina only and group 4 with no adjuvant. Likewise, mice were i.v. injected with anti-asialoGM-1 or anti-CD8β Abs (100 μg/100 μl) every week (17), challenged with B16D8 and fed with Spirulina (600 μg/600 μl) every other day. The tumor sizes were compared with those of control mice. Tumor volumes were measured at regular intervals using a caliper. Tumor volume was calculated using the formula: Tumor volume (cm^3) = (long diameter) x (short diameter) x (short diameter) x 0.4.

Statistical analysis

Student's t test was used to examine the significance of the data when applicable in animal studies. Comparisons with more than two groups were done using ANOVA with appropriate post hoc testing. Differences were considered to be statistically significant when $P < 0.05$.

ELISA, Flow cytometric (FACS) analysis of cell surface antigens

The levels of IFN-γ were determined by sandwich ELISA (Amersham Pharmacia Biotech, Buckinghamshire, UK) or the message levels assessed by quantitative PCR (23). The practical methods for FACS were described previously (24).

Quantitative RT–PCR

BMDCs were harvested at indicated time points (4 h) after treatment with Spirulina extracts (50μg/ml). The total RNA was extracted by RNeasy mini kit (Qiagen, Bothell, WA, USA). 0.5μg of total RNA was incubated at 70 for 5 min and then kept on ice for 2 min, and RT was performed as described previously (25). The following primers were used for quantitative PCR: IFN-β forward, 5'- CCAGCTCCAAGAAAGGACGA -3', and reverse 5'- CGCCCTGTAGGTGAGGTTGAT -3'. IP-10 forward 5'- GTGTTGAGATCATTGCCACGA -3', and reverse 5'-

GCGTGGCTTCACTCCAGTTAA -3'. β-Actin was used as an internal control to normalize reactions.

Assessment of in vitro cytolytic activity

The cytolytic activity of spleen NK cells was determined by ^{51}Cr assay as described previously (23). Effector lymphocytes were prepared from the spleen of intact C57BL/6 mice. A B16 subline (D8) or YAC-1 was used as a target cell. Target cells ($0.4x10^4$ cells/well) were coincubated with the effector splenocytes at the indicated lymphocyte to target (E/T) cell ratio (typically 1, 5 and 20) in V-bottom 96-well plates in a total volume of 200 μl of 0.5% BSA/RPMI-1640 medium at 37 °C. Four hours later, the liberated ^{51}Cr in the medium was measured using the scintillation counter. Specific cytolytic activity was obtained by the formula: Specific cytotoxic activity (%) =[(experimental ^{51}Cr activity - spontaneous ^{51}Cr activity)/(total ^{51}Cr activity – spontaneous ^{51}Cr activity)] x 100. Each experiment was done in triplicate to confirm reproducibility of the results, and representative results are shown. t test was used to examine the significance of the data.

Results

Tumor regression in mice having Spirulina

Retardation of tumor growth was observed in implant B16D8 tumor in mice when they were fed with Spirulina. The suppression of tumor growth by Spirulina was abrogated if the mice were treated with anti-asialoGM-1 Ab to eliminate NK cells (17) (Fig. 1A). The effect of Spirulina on tumor regression was somewhat variable in a mouse-to-mouse fashion, but was significant (n =8). The mouse group with Spirulina all survived 6 weeks after tumor challenge although some died in the control and NK-depleted group by 6 weeks. The results were confirmed with additional experiments, where NK activation occurred in mice in response to having Spirulina and disrupted by administration of anti-asialoGM-1 Ab (data not shown). We further confirmed that depletion of CD8+ T cells had virtually no effect on Spirulina-mediated antitumor activity (Fig. 1B). NK activation may have occurred in mice having Spirulina to retard tumor growth.

NK activation in mice given Spirulina

We confirmed that the hot water-extract of Spirulina induces NK activation by direct NK assay. Mice (wild-type) were orally administered with the extract of Spirulina or just saline (control) every other day for 2 weeks. The NK fraction of the spleen cells were prepared by MACS beads and cultured in vitro in medium only (Fig. 2A). NK-mediated cytotoxicity was determined using an NK-target, YAC-1. NK activation was enhanced in the group with Spirulina compared to those without Spirulina (Fig. 2A). Similar results were obtained with the B16D8 cells as targets (data not shown).

We next tested what molecular mechanisms participate in the Spirulina-mediated NK-enhanced activation. We used MyD88 -/- and TICAM-1 -/- mice to test the Spirulina NK-enhancing effect (Fig. 2B). In MyD88 -/-mice NK activation was not enhanced by administration of Spirulina, while in TICAM-1 -/- mice NK activation was enhanced by Spirulina as in wild-type mice. Hence, the TLR pathway involving MyD88, but not TICAM-1, participates in NK activation. NK cytotoxicity was already high in control mice having no Spirulina irrespective of disruption of the TLR pathways (Fig. 2B), suggesting that other factors than TLRs also participate in in vivo NK activation. We found that BMDCs in vitro prepared elicit NK-enhancing activity in response to the extract of Spirulina (Fig. 2C,D). Ability of Spirulina to drive NK activation in BMDCs was not abrogated with TICAM-1 -/- BMDCs but abrogated with MyD88 -/- BMDCs (Fig. 2D). Consistent with the result that MyD88 rather than TICAM-1 is crucial for Spirulina-mediated NK activation by BMDC, TICAM-1-dependent mediators, IFN-β and IP-10, were barely induced in Spirulina-stimulated BMDCs (Fig. 2E). Our interpretation of these results is that part of the in vivo NK-enhancing activity by Spirulina is attributable to the MyD88 pathway in BMDCs resulting in BMDC-NK reciprocal activation. Using this in vitro system, we tested if TLR2 and TLR4 are involved in the Spirulina NK-enhancing activity. TLR2/4-double deficient mice severely abrogated the response to Spirulina to reduce the Spirulina-mediated NK enhancing activity (Fig. 2C), although either one of TLR2 or 4 deficiency exhibited only marginal effect on NK-enhancing

activity by Spirulina. Thus, TLR2/4 and MyD88 participate in Spirulina-mediated BMDC-NK activation.

Additive tumor regression by BCG-CWS and Spirulina

BCG-CWS is an agonist of TLR2/4 to activate the MyD88 pathway, but does not activate NK cells (12,13), contrasting to the Spirulina extract. MyD88 may have two arms to drive CTL and NK cells in this context. BCG-CWS/Ag subcutaneously administered effectively matures antigen-presenting dendritic cells to induce tumoricidal CTL depending upon antigens selected (15). We next checked whether BCG-CWS/Ag and Spirulina elicit a synergistic effect on tumor regression in B16D8 melanoma-bearing mice (Fig. 3A). In this experiment, tumor burden was controlled for mice to survive >6 weeks after tumor challenge. Four mouse groups (n=20 each) were made to test if the tumor suppression activity depends on the host response to BCG-CWS/Ag and/or Spirulina in the same B16 implant system. Although either BCG-CWS/Ag or Spirulina alone exerted ability to regress implant tumor, the combination of both most effectively reduced tumor sizes ($p<0.05$) (Fig. 3A). Thus, Spirulina and BCG-CWS/Ag additively suppress tumor progression. Survival rate of mice challenged with B16D8 was also shown in Fig. 3B. The group treated with BCG-CWS and Spirulina survived long, since 45% of mice in this group were alive >9 weeks, by the time >80% of control mice died. Mice if treated with anti-asialoGM-1 Ab, all died by 9 weeks (Fig. 3B), suggesting the importance of NK cells for antitumor immune response and long survival.

Levels of IFN-γ in Spirulina/BCG-CWS-treated mice

IFN-γ is an effector that may be associated with prognosis of patients with BCG-CWS adjuvant immunotherapy (26). IFN-γ was secreted in the blood of mice with tumor burden if they were treated with BCG-CWS and Ag (Fig. 4). Either BCG-CWS/Ag or Spirulina alone slightly induced IFN-γ. The levels of IFN-γ induced by Spirulina appeared low in mice compared to human volunteers (11). Tumor antigen had no effect on the level of Spirulina-mediated IFN induction (data not shown). Notably, significantly high levels of IFN-γ were detected in mice treated with both BCG-CWS/Ag

and Spirulina (Fig. 4). Skin reaction reflecting BCG hypersensitivity was observed in the relevant group (data not shown). No or less skin reaction was observed in the group with BCG-CWS/Ag and Spirulina. Spirulina alone did not induce skin reaction.

Rae-1-positive B16 cells were eliminated by Spirulina

B16D8 cells in implant tumor of C57BL/6 mice consisted of Rae-1-positive and –negative cells. The cells were inoculated into the mice which were fed and/or treated with the material indicated (Fig. 5). Implanted B16 tumor cells were extracted from the tumor-bearing mice five weeks after tumor challenge, stained with the indicated Abs and analyzed by FACS (Fig. 5), where mean fluorescence intensities (MFI) are shown in the insets. In the group having no Spirulina, tumor cells consisted of Rae-1-positive and –negative populations, whereas in the group having Spirulina, Rae-1-positive population was selectively diminished. The group treated with Spirulina and anti-asialoGM-1 Ab possessed the Rae-1-positive population. Other markers including MHC class I and class II were neither detectable nor different among the groups. Although other NK-activation ligands were detected in message levels, they still remained in the Rae-1-negative cells (data not shown). Thus, Rae-1-positive tumor cells are selectively eliminated by Spirulina-derived NK cells in this mouse system.

This issue was confirmed using an in vitro assay. NK cells reciprocally activated by Spirulina-treated BMDCs damaged B16D8 cells (Fig. 6A). The NK-mediated B16D8 killing was largely blocked by the addition of anti-NKG2D Ab (Fig. 6A). Without BMDCs, Spirulina extract treatment barely activated NK cells (data not shown). Thus, NK-mediated B16D8 killing was attributable to interaction between tumor cell Rae-1 and NK cell NKG2D, which situation was supported by Spirulina-dependent maturation of BMDC: there is no direct route for Spirulina-mediated NK activation.

Finally, Spirulina-mediated B16D8 growth was largely abrogated in MyD88 -/- mice (Fig. 6B). Antitumor NK activation predicted in this study may actually occur in tumor-bearing mice through having Spirulina.

Discussion

We demonstrated that tumor growth is retarded in mice having Spirulina. Unique points in this study are that 1. Rae-1-positive tumor cells are selectively eliminated in Spirulina-fed mice, 2. marked tumor regression is observed in mice with combinational administration of BCG-CWS/Ag and Spirulina. Synergistic effect on tumor regression and increase of serum IFN-γ level was observed in mice by combination treatment with BCG-CWS/Ag and Spirulina. BCG-CWS/Ag induces CTL via TLR2/4 in myeloid DCs in a MyD88-dependent manner (15). In this study, Spirulina activates NK cells also via TLR2/4 in BMDCs in a MyD88-dependent manner. These reports suggest that simultaneous attack by CTL and NK more effectively regress tumor cells in mouse tumor implant models.

Our in vitro findings on Spirulina allowed us to interpret that the Spirulina extract activates the MyD88 pathway in BMDCs to evoke activation of mouse NK cells (Fig. 2). What mechanisms activate the MyD88 pathway to drive NK cells in Spirulina-stimulated BMDCs is an intriguing issue, since other TLR2/4 ligands mainly engage CTL induction, but not NK activation, via the MyD88 pathway in BMDCs (27,28). Difference in downstream signal events may cause the different outcomes of NK/CTL induction in BMDCs. Studying the Spirulina-activated MyD88 pathway will be an important issue to clarify how it is possible to have two TLR2/4 agonists activate NK and CTL.

There appear several routes for activation of NK cells. The BMDC-mediated NK activation serves an important route for NK-mediated tumor clearance (29-31). We demonstrated that the TICAM-1 (TRIF) pathway in BMDCs participates in the DC-NK reciprocal activation (17,27,32). In contrast, most of the non-DNA/RNA adjuvants currently available are originated from bacteria and activate the MyD88 pathway of TLR2 and/or TLR4 in DCs, but they barely induce NK activation (17,28). In this context, BCG-CWS expressed high tumor-killing activity (Fig. 3A), but this activity was barely abrogated by administration of anti-asialoGM-1 Ab (data not shown). Furthermore, Spirulina appears to differ from LPS in its adjuvant activity, since LPS simultaneously activates the MyD88 and

TICAM-1 pathways to evoke the systemic cytokine storm. The Spirulina extract is unique because it has an ability to activate NK cells without inducing type I IFNs.

Spirulina-mediated IFN-γ production is greatly enhanced in mice by simultaneous administration of BCG-CWS/Ag, similar to humans (33). How Spirulina participates in IFN-γ production is a coming question. Spirulina ultimately activates T and NK cells in mice, although IFN-γ is poorly induced in mice compared to human volunteers with only Spirulina (11). Direct addition of Spirulina to splenic lymphocytes neither results in NKG2D up-regulation nor induces enhanced B16D8 cell killing (data not shown), suggesting that cell populations other than DCs in the spleen barely participate in this event. There is a paper reporting that the MAPK pathway of BMDCs participates in promotion of NK activation by BMDCs (34). If this is the case, NK-enhancing effect by Spirulina in human patients can be evaluated in the mouse model and IFN-γ production is a good marker for NK activation.

Previous studies on patients with cancer suggested that Spirulina constituents serve as protective agents against oral cancers (35) in terms of tumor progression and metastasis (36), although the results are of non-randomized trials. These reports were reminiscent of the antitumor function of Spirulina, some of which would be derived from β-carotene, an effective antioxidant (37). Indeed, the serum levels of β-carotene are consistently low in patients with cancer (37,38). Our present study may offer additional evidence that NK activation is a representative of the Spirulina-mediated antitumor immunity.

However, a point remains unsettled that the constituents of Spirulina taken up via the intestine and colon do not always correspond to those of the extract of Spirulina. In vitro studies where Spirulina was added to NK cells or BMDCs appear not to be simply comparable with the in vivo studies using mice. In other reports, Spirulina orally taken significantly reduced IL-4 levels in individuals with allergic rhinitis by randomized double-blinded trial (39). Another report suggested that Spirulina enhances IgG1 and IgA production but not IgE production by modulating the mucosal immune system (9). Spirulina may skew the Th2 polarization to a Th1-like

state in allergic patients. We should identify the Spirulina constituents associated with activation of mucosal immunity and absorbed into the circulation.

Since cancers are usually established circumventing the host immune system including NK and CTL, tumor cells generally possess poor immunogenicity by expressing low levels of MHC class I and ligands for NK-activating receptors including NKG2D (40). It is notable that these receptor levels are regulated by IFN-γ. It has been reported that during BCG adjuvant therapy the increased serum level of IFN-γ 18 h after s.c. injection of BCG-CWS is a marker for evoking of the innate immune response (26). In mouse experimental models using syngeneic transplantable tumors, MHC class I-expressing tumor cells were selectively eliminated by BCG-CWS/Ag s.c. injection (15), and the serum levels of IFN-γ was increased in this case of Spirulina, too. Nevertheless, tumor cells with low MHC expression remain surviving resulting in MHC-negative tumor progression. This study clearly shows that tumor growth is suppressed in an MHC-negative/Rae-1-positive population of tumor cells by treatment of tumor-implant mice with Spirulina, which can induce NK activation to damage tumor cells via NKG2D receptors.

In human cancer patients receiving BCG-CWS therapy, tumor cells are not completely disappeared in the primary region (41), although patients' QOL scores are increased in response to the BCG-CWS therapy. Similar observations were reported in patients with bladder cancer who selected BCG immunotherapy (42). Growing the low MHC tumor cells may account for the incomplete remission of tumor in cancer patients.

We offer the possible immune therapy in combination with BCG-CWS and Spirulina in this communication. Additive tumor cytotoxicity based on BCG-CWS/Ag and Spirulina suggests that they elicit different effectors, putative CTL and NK. Their combinational function merely targets the MyD88 pathway and is clearly distinct from that of LPS that induces toxic shock. Although what constituents of the Spirulina extract are responsible for NK enhancement, and why Spirulina and BCG-CWS differentially activate the TLR2/4-mediated MyD88 pathway in DCs should be further investigated, this is the fist report predicting that the combination of

BCG-CWS/Ag and Spirulina is applicable to immunotherapy for patients with tumor mass of variable MHC levels. We favor the premise that Spirulina is a candidate of NK activator applicable to cancer patients by oral usage.

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Figure legends

Fig. 1. AsialoGM-1 Ab-sensitive retardation of MHC-low tumor growth by oral administration of Spirulina.

A) Spirulina induces antitumor NK activation. C57BL/6 mice were separated into three groups (n=8): one pretreated with anti-asialoGM-1 Ab and two groups with control saline, and except one control, fed with Spirulina from day 0, when B16 D8 subline was s.c. inoculated. Tumor volume was measured at timed intervals as described in the Methods and statistical analysis was performed as described (15). One mouse with saline only and one mouse with Spirulina and anti-asialoGM-1 Ab died of tumor after 5 weeks. We finally applied to statistical analysis live 7 mice at the 5-week point of the two groups and declared the significance ($p<0.05$). B) CD8+ T cells barely participate in Spirulina-mediated retardation of tumor growth. Wild-type C57BL/6 mice were grouped (n = 5). Spirulina extract (600 μg/600 μl) (●, ▲) or control saline (○) was orally administered to them every other day from day −14. One group (▲) was pretreated with anti-CD8β Ab as described in the text. Then, B16D8 cells ($6x10^5$/head) were subcutaneously inoculated into the mice at day 0. Tumor volume was measured at indicated timed intervals and statistical analysis was performed as described (15).

Fig. 2. Orally administered Spirulina enhances NK activation in mice.

A) YAC-1 killing activity of spleen NK cells harvested from mice with or without Spirulina. Spirulina extract (600 μg/600 μl) was orally administered into mice every other day. 2 weeks later, spleen cells were harvested to isolate NK cells by MACS beads from wild-type mice. NK activity against YAC-1 or B16D8 cells (not shown) were determined at the indicated E/T ratio. B) Participation of MyD88 in enhanced NK activation by Spirulina. NK cells were prepared from wild-type, TICAM-1 -/- or MyD88 -/- mice which had been treated with saline or Spirulina extract as in panel A. NK cytotoxic activity was determined using YAC-1. C) Spirulina effect on BMDC-NK activation was abrogated with TLR2/4 -/- BMDCs. BMDCs from wild-type and TLR2/4-double deficient mice were stimulated with Spirulina extract for 4 h and mixed with NK cells for 24 h. The mixture

was incubated with ^{51}Cr-labelled target B16 cells for 4 h at the E/T ratio indicated (left panel). In the right panel, BMDCs of the indicated KO mice were admixed with NK cells as in the left panel. NK activity was determined using ^{51}Cr-labelled B16 target at the fixed E/T ratio (1/25). % cytotoxicity was determined as in panel A. Only slight abrogation of the Spirulina-mediated NK activation was observed in either TLR2 -/- or TLR4 -/- BMDCs under the same conditions (data not shown). D) Spirulina acts on BMDC and augments MyD88-mediated NK reciprocal activation by BMDC. BMDCs with TICAM-1 -/- (left panel) and MyD88 -/- (right panel) were stimulated with Spirulina extract for 4 h and mixed with NK cells for 24 h. The mixture was incubated with ^{51}Cr-labelled target B16 cells for 4 h at the E/T ratio indicated. % cytotoxicity was determined as in panel A. E) The mRNA levels of IFN-β and IP-10 in BMDCs were determined by quantitative PCR 4 h after Spirulina stimulation. These experiments were performed 3 times and similar results were obtained. Representative ones are shown.

Fig. 3. Implant tumor retardation by BCG-CWS and Spirulina in mice.

A) C57BL/6 mice were separated into four groups (n=20). Group 1 with saline only, group 2 with BCG-CWS/B16 Ag, group 3 with Spirulina and group 4 with BCG-CWS/Ag and Spirulina. BCG-CWS/Ag was administered four times from day –21 to day 0 (15), while Spirulina was fed from day –21 to the end of the survey (see the inset). B16D8 subline was s.c. inoculated at day 0. Tumor volume was measured at timed intervals and statistical analysis was performed as described (15). Panel B: Mice were grouped into the four (n=20) as indicated in the panel as in panel A, and the effect of asialoGM-1 Ab on the survival of tumor-implant mice was analyzed. The survival rate was plotted for each group of mice.

Fig. 4. Serum level of IFN-γ in tumor-bearing mice having BCG-CWS and/or Spirulina.

Mice were treated with BCG-CWS/B16 Ag and/or Spirulina and inoculated with B16 tumor at day 0 as in Fig. 3. 48 h after tumor implanting, mouse sera were obtained from their tails and the levels of IFN-γ were