Enhancement of Natural Killer Cell Activity in Healthy Subjects by Immulina®, a *Spirulina* Extract Enriched for Braun-Type Lipoproteins

Authors Claus Henrik Nielsen[1], Premalatha Balachandran[2,3], Ole Christensen[1], Nirmal D. Pugh[2,3], Hemlata Tamta[2,3], Kenneth J. Sufka[3,5,6], Xiangmei Wu[2,3], Anette Walsted[7], Michelle Schjørring-Thyssen[8], Christian Enevold[1], David S. Pasco[2,3,4]

Affiliations The affiliations are listed at the end of the article

Key words
- *Arthrospira platensis*
- Phormidiaceae
- *Spirulina*
- natural killer (NK) cells
- Braun-type lipoproteins
- Immulina
- clinical trial

Abstract

Immulina®, a commercial extract of *Arthrospira (Spirulina) platensis* is a potent activator of THP-1 monocytes and CD4+ T cells *in vitro* and enhances several immunological functions in mice. We further characterized Immulina® by determining that Braun-type lipoproteins are responsible for a major portion of the *in vitro* monocyte activation exhibited by this material. In order to understand the effect of Immulina® on NK cell activity, a pilot study was conducted on ten healthy North American individuals who supplemented their diet with Immulina® (400 mg/day) for seven days. We observed a 40% average increase in the killing of K562 tumor cells by NK cells ($p < 0.01$) after Immulina® supplementation. In a separate placebo-controlled, crossover study involving 11 healthy Danish subjects, we observed increased mRNA expression of the NK cell marker NKG2D by 37% ($p = 0.02$) and by 55% ($p = 0.0003$) after administration of Immulina® (200 mg and 400 mg per day, respectively) for seven days. The mRNA expression of the NK- and T-cell marker perforin increased by 75% ($p = 0.008$) after administration of 400 mg Immulina® per day. Both markers displayed significant dose-dependent effects ($p = 0.0003$ and $p = 0.02$, respectively). The ratio between $CD56^{bright}$ and $CD56^{dim}$ NK cells was not affected by Immulina® administration. In summary, two independent studies showed enhancement of NK cell activity following administration of Immulina® for seven days.

Abbreviations

LDH:	lactate dehydrogenase
PMN:	polymorphonuclear cell

Supporting information available online at http://www.thieme-connect.de/ejournals/toc/plantamedica

received February 17, 2010
revised May 5, 2010
accepted May 12, 2010

Bibliography
DOI http://dx.doi.org/10.1055/s-0030-1250043
Published online June 17, 2010
Planta Med 2010; 76: 1802–1808 © Georg Thieme Verlag KG Stuttgart · New York · ISSN 0032-0943

Correspondence
David S. Pasco, Ph.D.
National Center for Natural Products Research
School of Pharmacy
University of Mississippi
University Ave
University, MS 38677
USA
Phone: + 1662915 7130
Fax: + 1662915 7062
dpasco@olemiss.edu

Introduction

Arthrospira platensis (Nordstedt) Gomont, Phormidiaceae (previously called *Spirulina platensis* and commonly called *Spirulina*) has a long history of use as food [1]. It has also been consumed by humans for many years as a health drink or pills/tablets without any toxicological effects [2] and its safety has been well established. The immune-enhancing properties of *Spirulina* or its components have been demonstrated in various animal models such as in chickens [3,4], dogs [5] and mice [6].

Immulina® is a commercial extract of *Spirulina platensis* that is standardized by biological activity. Our earlier studies have shown that the active components of Immulina® were potent activators of NF-κB and induced mRNAs encoding IL-1β and TNF-α [7] as well as the chemokines IL-8, MCP-1, MIP-1β and IFN-γ inducible protein-10 [8] in THP-1 monocytes. Our later work showed that the components within Immulina® that activate monocytes do so predominantly through a TLR2-dependent process [9]. Blocking antibodies to TLR2 and CD14 blocked activation of NF-κB in human THP-1 monocytes while TLR4 antibodies had no effect. In addition, cells transfected with plasmid constructs expressing both TLR2 and CD14 exhibited NF-κB activation when treated with Immulina® while cells expressing TLR4, CD14 and MD-2 did not support activation. That study also demonstrated that oral consumption of Immulina® by mice for four to five days enhanced the *ex vivo* production of IgA and IL-6 from Peyer's patch cells and IFN-γ from spleen cells [9]. This indi-

cated that dietary Immulina® impacted both gut mucosal and systemic immune function.

In addition to enhancing innate immune functions, a recent study suggests that Immulina® also impacts adaptive immune responses. Consumption of Immulina® by 11 healthy male volunteers caused an immediate, but temporary increase in CD4+ T-helper cell proliferation after *ex vivo* exposure to the recall antigen *C. albicans* [10]. B cell proliferation was also enhanced by *C. albicans* at eight days but was reduced by 56 days. *Ex vivo* production of the Th1 cytokines TNF-α, IL-2, and IFN-γ as well as the Th2 cytokines IL-6 and IL-5 was also enhanced by *C. albicans* during the first week of Immulina® ingestion. These results suggest that Immulina® ingestion has a temporary priming effect on adaptive immune responses to antigenic stimuli that is most likely related to its strong proinflammatory effect on innate immune cells.

NK cells represent important early effector cells of the innate immune system and can lyse virally infected and malignant cells without prior sensitization [11,12]. Early studies in chickens showed that dietary *Spirulina* enhanced NK cell activity by two-fold [3]. A study using a small number of healthy human volunteers suggested that ingestion of a hot water extract of *Spirulina* influenced NK cell function [13]. The production of IFN-γ by NK cells incubated *in vitro* with either IL-12 or IL-12 plus IL-18 was substantially enhanced in all four individuals that ingested this extract for two months. Using a different set of four subjects, NK cell cytotoxic activity was enhanced in two of the individuals after taking this extract for two or four weeks.

In light of the results obtained in the human NK cell study above, we investigated the effect of short-term (one week) Immulina® ingestion on NK cell activity in two groups of healthy adults. In addition, the levels of several NK cell molecular markers were determined to further characterize the action of Immulina® on this important innate immune cell. We also identified the main component within this extract responsible for the TLR2-dependent activation of innate immune cells *in vitro*.

Materials and Methods

▼

Materials

Histopaque-1077 was purchased from Sigma-Aldrich. Human erythroleukemic K562 cells and human monocytic THP-1 cells were obtained from American Type Culture Collection. Immulina® is available in capsule form and these capsules are manufactured and marketed by Nordic Immotech A/S. This extract represents a 10 to 15% yield from spray dried *Spirulina platensis* and is standardized by biological activity using a human THP-1 monocyte activation assay to ensure consistent activity of the extract from batch to batch. Batch number of capsules for Study 1 was 8638313 and for Study 2 it was 8837210. Voucher specimens for extract and raw material for both studies were deposited in the NCNPR repository at the University of Mississippi.

Research design

Study 1 (Pilot study): The Institutional Review Board of the University of Mississippi approved this study and all volunteers signed informed consent forms. Ten normal healthy volunteers (7 males and 3 females, ages 22–57 years with a mean of 37 years), who had no serious physical complaints, were not pregnant or did not recently donate blood, were recruited for a pilot study at The University of Mississippi. Subjects taking prescription medications, botanicals, yogurt or vitamin supplements of any kind were excluded from participation in the study. The individuals who fulfilled the eligibility criteria were asked to take Immulina® 2 times a day with meals, in the form of capsules (200 mg per capsule) for the period of 7 days. Peripheral blood samples were drawn from subjects by venipuncture in the morning during the spring of 2007.

Study 2 (double-blind, placebo-controlled): The Institutional Review Board of the Regional Ethics Committee for the Capital of Denmark (reference number H-D-2008-014) approved this study and all volunteers signed informed consent forms. To confirm the results of the pilot study, twelve healthy volunteers (5 males and 7 females, ages 26–69 years with a mean of 52 years) fulfilling the above-mentioned criteria were included in a study in Copenhagen, Denmark, during the spring of 2009. The sample size was determined on the basis of the pilot study (Study 1). Exclusion criteria were chronic disease, intake of prescription medications, botanicals, yogurt or vitamin supplements of any kind. One subject was subsequently excluded from the analyses, due to intake of nonsteroid anti-inflammatory drugs during the study.

A crossover design was chosen, in which the subjects received Immulina® capsules 1 × 200 mg, Immulina® capsules 2 × 200 mg, or placebo capsules for one week. All participants received each treatment with an interval of 3.5 weeks, in a random sequence. They were enrolled by the author MS-T, who also determined the allocation sequence but took no part in the laboratory assessments. The treatment of the participants was blinded to the laboratory staff assessing the outcomes.

Statistics

For Study 1, one-tailed, repeated measures t-test was used to identify significant changes in NK cell activity pre- versus post-Immulina® treatment and p values less than 0.05 were considered significant.

For Study 2, to allow for day-to-day variation and possible carry-over effects, all post-Immulina® measurements were related to the average values of four measurements performed after no intake of Immulina® (before and after placebo, before 200 mg and before 400 mg Immulina®). The observed data were log-transformed prior to analysis to fulfill the assumption of normality, and were subsequently analyzed by an ANOVA model with day, dose and previous dose included as fixed effects and subject included as random effect. Since only the contrasts between 200 and 0 mg; and 400 and 0 mg were tested, no correction for multiplicity was applied. The statistical software employed was SAS v.9.1.3 (SAS Institute). P values less than 0.05 were considered significant.

Braun-type lipoprotein characterization and identification

The identification of Braun-type lipoproteins in Immulina® and the contribution of these compounds to the *in vitro* activation of THP-1 monocytes were determined using various biochemical techniques as described previously [14].

THP-1 monocyte assay

This assay was used for biological standardization of Immulina® and for determination of activity of samples from the lipoprotein characterization experiments. The THP-1 human monocyte cell line was transfected with a luciferase reporter gene construct containing two copies of NF-κB motif from HIV/IgK as described previously [7]. Response to a sample is reported as a percentage relative to maximal activation of NF-κB by LPS (10 µg/mL, *E. coli*,

serotype 026:B6; Sigma). Immulina® material used for these studies exhibited an EC_{50} of 25 ng/mL while that of the synthetic Braun-type lipoprotein, Pam3CSK4 was 0.1 ng/mL (InvivoGen).

Supporting information

The PBMC isolation methods, NK cell cytotoxicity assay, RT-qPCR and flow cytometry techniques and the details about primers (**Table 1S**) and antibodies used in this study are available as Supporting Information.

Results

In Study 1, ten healthy individuals were supplemented with Immulina® capsules for 7 days. Adverse side effects were monitored daily during the study period, but none were reported. The effects of Immulina® supplementation on tumoricidal activity of the PBMC fraction, against K562 cells are summarized in **Fig. 1**. Nine of the ten participants showed an elevation in their NK cell cytotoxic activity. Consistent with these observations, a comparison between pre- and post-Immulina® supplementation for all subjects showed a significant increase in the % tumor cell death, $t(9) = 3.88$, $p < 0.005$. When adjusted for individual differences in baseline level, Immulina® supplementation resulted in an average increase in NK cell cytotoxic activity of 40% and this effect was also statistically significant, $t(9) = 3.41$, $p < 0.01$. *In vitro* studies with this cell target showed that purified NK cells exhibited cytotoxic activity, while PBMC depleted for NK cells did not. In addition, Immulina® extract at concentrations from 25 to 100 µg/mL did not increase target cell killing by either purified NK cells or by PBMC devoid of NK cells *in vitro* (data not shown).

The findings described above prompted us to undertake a placebo-controlled, double-blind study (Study 2) on the effects of Immulina® in a cohort of twelve healthy individuals, and in a different laboratory. Each individual received 400 mg Immulina®, 200 mg Immulina®, or placebo for 7 days, in a random sequence, 3.5 weeks apart. None of the participants suffered any adverse effects during the study. When adjusted for individual differences in baseline level, tumoricidal NK-cell activity using a PBMC : K562 ratio of 5 : 1 was increased −10%, 83% and 54% with placebo, 200 mg and 400 mg Immulina®, respectively (**Fig. 2a**). Using a PBMC : K562 ratio of 10 : 1 the corresponding increases were −4%, 42% and 48%, respectively (**Fig. 2b**). Although a paired t-test revealed a significant increase in NK cell cytotoxic activity in the 400 mg dose ($p = 0.03$) using the PBMC/target ratio of 5 : 1 when the highly variable 200 mg treatment condition was removed, analysis of variance of both tested PBMC/target ratios failed to show significant treatment effects when adjusted for day-to-day variation and possible carryover effects.

We next tested whether treatment with Immulina® produced significant differences between pretrial and posttrial levels of mRNAs for three NK cell markers: i) the activating receptor NKG2D, a potent stimulatory immunoreceptor that is expressed on NK cells, NKT cells, γ/δ^+ T cells and $CD8^+$ T cells [15]; ii) the adhesion molecule CD56, also known as neural cell adhesion molecule, expressed by NK cells and activated T cells, as well as by cells within the nervous system and skeletal muscle cells [16]; and iii) perforin, a cytolytic protein found in the granules of $CD8^+$ T cells and NK cells [17]. When adjusted for day-to-day variation, **Table 1** shows that NKG2D expression was 37% higher after intake of 200 mg Immulina® per day ($p = 0.02$) and 55% higher after intake of 400 mg Immulina® per day ($p = 0.0003$) than after intake of 0 mg per day. No significant increase was observed for CD56 expression, while we found a 75% increase in the expression of perforin after intake of 400 mg Immulina® per day ($p = 0.008$) and a borderline-significant increase (by 58%) after intake of 200 mg ($p = 0.06$), as compared to the average levels after no intake of Immulina®. A significant dose-dependency was observed for the expression of both NKG2D ($p = 0.0003$) and perforin ($p = 0.02$). We observed no carryover effect between the treatment cycles with respect to the mRNA expression of NKG2D ($p = 0.69$), CD56 ($p = 0.99$) or perforin ($p = 0.75$), indicating that the effect of Immulina® waned within 3½ weeks.

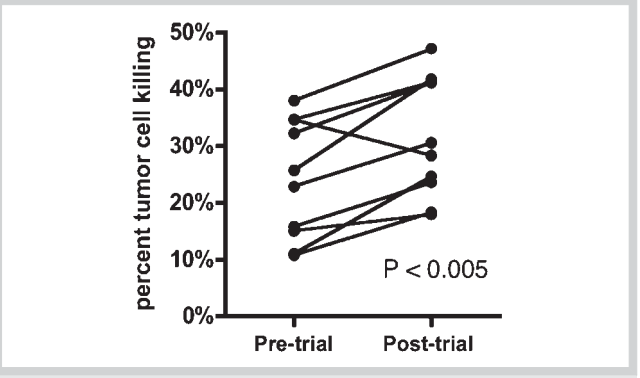

Fig. 1 Effect of Immulina® supplementation on cytotoxic activity of NK cell fraction in PBMC cultures. Healthy individuals (n = 10) consumed Immulina® capsules (400 mg/day) for seven days. Peripheral blood was collected before and after supplementation with Immulina®. PBMC were isolated as described in the Materials and Methods section as a source of NK cells. Tumor cell killing activity was measured via the lysis of K562 target cells by PBMC using an effector : target cell ratio of 50 : 1. Each point represents the mean of quadruplicate determinations for each individual expressed as percent tumor cell killing by NK cells at pretrial and posttrial time points.

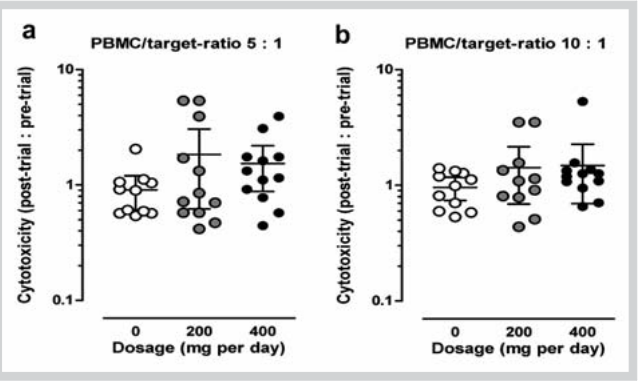

Fig. 2 Immulina®-mediated changes in NK-cell cytotoxicity in a placebo-controlled study. Healthy individuals consumed placebo or Immulina® capsules (200 mg/day or 400 mg/day) for seven days in a crossover design with 3.5 weeks interval between the administrations. Tumor cell killing activity was measured via the lysis of K562 target cells by PBMC, using an effector: target cell ratio of 5 : 1 (**a**) or 10 : 1 (**b**). The data are shown as percent tumor cell killing after 7 days of Immulina® consumption divided by percent tumor cell killing prior to consumption, assessed in duplicate for each individual. Bars and error bars represent mean and 95% confidence intervals for 11 applicable participants (one was excluded due to administration of nonsteroid anti-inflammatory drugs).

Table 1 Influence of Immulina® on the expression of mRNA encoding NK-cell associated molecules.

Gene product	Gene	Dosage	Ratio with 95% CI	P value[1]
NKG2D	KLRK1	200 mg versus 0 mg	1.37 (1.05–1.79)	**0.02**
		400 mg versus 0 mg	1.55 (1.24–1.95)	**0.0003**
		200 mg versus 400 mg	1.13 (0.92–1.39)	0.24
CD56[2]	NCAM1[2]	200 mg versus 0 mg	1.29 (0.36–4.64)	0.69
		400 mg versus 0 mg	1.57 (0.56–4.44)	0.38
		200 mg versus 400 mg	1.22 (0.48–3.13)	0.67
Perforin	PRF1	200 mg versus 0 mg	1.58 (0.97–2.58)	0.06
		400 mg versus 0 mg	1.75 (1.16–2.63)	**0.008**
		200 mg versus 400 mg	1.11 (0.76–1.61)	0.60

Expression of mRNA encoding NKG2D, CD56 and perforin from the genes KLRK1, NCAM1and PRF1, respectively, was measured by real-time polymerase chain reaction in isolated PBMC before and after administration of placebo, Immulina® 200 mg/day, or Immulina® 400 mg/day for one week. The housekeeping gene beta-actin was used as a reference gene in all measurements. The ratios represent the indicated post-Immulina® value divided by the average of four measurements where the individuals received 0 mg Immulina® (pre-placebo, post-placebo and pre-200 mg Immulina® and pre-400 mg Immulina®), and are shown as the mean of 11 individuals with lower and upper limits of 95% confidence intervals (CI). One individual was excluded from analysis due to intake of nonsteroid anti-inflammatory drugs during administration of placebo. [1] Probability for the ratio = 1 as calculated by an ANOVA model using pairwise comparisons of log-transformed data; values < 0.05 are shown in bold. [2] A further two outliers in the placebo column (with ratios 0.00 and 3522, respectively, as compared to beta-actin), and one outlier in the 200 mg/day column (ratio 0.00 as compared to beta-actin) were excluded from the analysis of CD56 expression

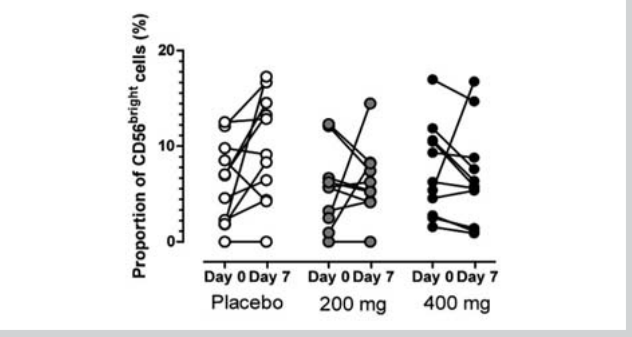

Fig. 3 Immulina®-mediated changes in the proportion of $CD56^{bright}$ NK cells. On the basis of staining with APC-conjugated anti-CD56, NK cells were subdivided into $CD56^{bright}$ and $CD56^{dim}$ subsets, and the change in the proportion of $CD56^{bright}$ cells between day 0 and day 7 after consumption of placebo, Immulina® (200 mg/day) and Immulina® (400 mg/day), as assessed by flow cytometry, is shown for 11 subjects. One outlier was removed from the 200 mg/day cycle.

Approximately 10% of NK cells are known to express high levels of CD56 and are commonly referred to as $CD56^{bright}$ NK cells [18]. Unlike the more preponderant $CD56^{dim}$ NK-cell subset, the $CD56^{bright}$ subset lacks inhibitory killer cell immunoglobulin-like receptors, and expresses a distinct repertoire of chemokine receptors and adhesion molecules. They are regarded as less cytotoxic than $CD56^{dim}$ NK cells, but are more efficient cytokine producers. We did not observe any influence of Immulina® consumption on the proportion of $CD56^{bright}$ and $CD56^{dim}$ NK cells (**Fig. 3**); neither did Immulina® significantly affect the overall numbers of circulating NK cells, NKT cells and T cells, or the expression of the activity marker CD69 on the surface of the individual cell populations (data not shown).

We previously showed that NF-κB activation in the human monocyte cell line THP-1 by the high molecular weight fraction of the Immulina® extract was dependent on CD14 and TLR2 [9]. This fraction makes up from 25 to 30% of the Immulina® extract and is responsible for the majority of the activity of this extract. Since *Spirulina* is a prokaryotic cyanobacteria and since Braun-type lipoproteins from both gram-positive and gram-negative bacteria potently activate NF-κB through a CD14- and TLR2-dependent process [19–21], we employed several established biochemical methods to determine if this bacterial component contributed to the activity of the Immulina® extract. **Fig. 4a** shows that protein was part of the active molecule since proteinase K treatment of the Immulina® extract (black bars) decreased the overall size of the active component as compared to the untreated extract (white bars) when fractionated on an SDS polyacrylamide gel. The activity of the untreated (C) and proteinase K treated (PK) Immulina® extract before gel electrophoresis were similar indicating that the protein component was not required for activity, similar to other characterized bacterial lipoproteins [19,21]. The results presented in **Fig. 4b** show that the activity present in the Immulina® extract is completely abrogated by removal of glycerol bound fatty acids by treatment with lipoprotein lipase (diacylglycerol lipase). This result together with the results presented in **Fig. 4a** suggest that lipoproteins are responsible for the majority of the activity within the Immulina® extract. Bacterial lipoproteins of the Braun type are produced by both gram-negative and gram-positive bacteria and are thought to be unique to prokaryotes. These lipoproteins contain a diacylglycerol moiety that is linked by a thioether bond to the N-terminal cysteine of the peptide chain [22]. Evidence that the active lipoproteins detected in the Immulina® extract were indeed of the Braun type is indicated by the detection of the modified amino acid 2,3 dihydroxypropylcysteine [23] using reverse phase-HPLC (Peak A, **Fig. 4c**).

Discussion

The results of Study 1 presented here indicate that supplementation of the diet with Immulina® (a commercially available, standardized extract of *Spirulina platensis*) can lead to enhanced *ex vivo* tumoricidal activity of NK cells. The crossover study (Study 2), showed a similar tendency at the 400 mg dose, but statistical significance was not reached when including the highly variable results of the 200 mg dose and day-to-day variation in the analysis. However, Study 2 showed an effect of Immulina® on the expression of mRNA encoding the NK cell-associated molecules NKG2D (at both dosages) and perforin (at 400 mg Immulina® per

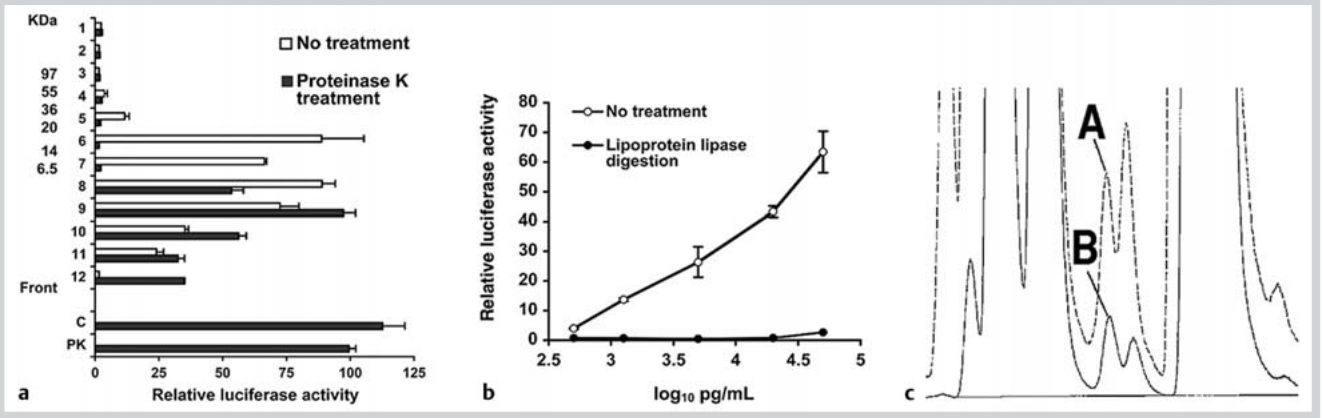

Fig. 4 The majority of activity detected in the Immulina® extract is due to Braun-type lipoproteins. **a** Immulina® extract was dissolved in 1% octylglucoside and treated with proteinase K (black bars) or left untreated (white bars). Samples (100 µg) were heated at 98 °C for five minutes in 1.5% SDS containing buffer and fractionated on a 16.5% Tris-Tricine gel. Individual lanes were cut into 0.5-cm sections, extracted with octylglucoside and evaluated for activity in THP-1 human monocytes transfected with a NF-κB luciferase reporter plasmid. The black bars labeled C and PK represent the activity of the untreated and proteinase K treated samples respectively, before fractionation on the gel. Values are the average of duplicate determinations in a representative experiment that was repeated three times. **b** Immulina® extract was dissolved in 1% octylglucoside and samples were then incubated at 37 °C for 16 hours with lipoprotein lipase. Control samples (without lipoprotein lipase) were run under identical conditions. Luciferase activity was determined in THP-1 human monocytes transfected with a NF-κB luciferase reporter plasmid. Values are the average of duplicate determinations in a representative experiment that was repeated twice. Positive control values for synthetic lipoprotein Pam3CSK4 run at 0.5 ng/mL were 109.2 ± 1.28 (untreated) and 12.4 ± 0.53 (lipoprotein lipase treated). **c** Identification of 2,3-dihydroxypropylcysteine, a structural component of Braun-type lipoproteins. Lipoproteins were extracted from Immulina® extract using 4% SDS and the low-molecular weight components (less than 5000 Daltons) were removed by ultrafiltration. The lipoproteins in the high-molecular weight fraction were treated with proteinase K to remove the bulk of the protein component and the lipopeptide moieties were partitioned into phenol. The phenol was removed by freeze drying and the active sample and the synthetic lipoprotein Pam3CSK4 (used as a standard) were hydrolyzed using methanesulfonic acid. Hydrolysates were analyzed using a Hewlett Packard Amino-Quant System. The chromatogram section shown represents separation of active Immulina® fraction (dashed line) and active Immulina® fraction spiked with standard (solid line) using reverse-phase HPLC with fluorescence detection after being derivatized with *o*-phthalaldehyde. Peak A (2,3-dihydroxypropylcysteine) eluted with a retention time (7.20 min) identical to the standard and identification was further supported by spiking of the active fraction with the standard (Peak B).

day), which supported the conclusion of Study 1 of an effect of Immulina® on NK cells. We observed no significant change in CD56 expression, which may be due to either distinct turnover kinetics of this molecule on NK cells, or to a diluting effect on mRNA by the subset of T cells expressing this molecule [16], since the mRNA analysis were made on PBMC, and not isolated NK cells. For the same reason, we cannot exclude that the increase in perforin expression, in particular, was partly due to an effect of Immulina® on T cells. However, our data showed that NK cells were solely responsible for the tumor cell killing.

We also show that the principal innate immune enhancing component of the Immulina® extract is Braun-type lipoproteins. This finding is consistent with our previous studies demonstrating that NF-κB activation by the Immulina® extract was via a TLR2-dependent pathway [9]. This is also the first report indicating that lipoproteins of the Braun type, a principal TLR2 ligand [21], contribute to the immune enhancing properties of *Spirulina*. Since activation of innate immune cells with Immulina® extract is TLR2-dependent rather than TLR4-dependent, this extract would impact TLR-containing immune cells similar to bacterial components from gram-positive rather than gram-negative bacteria. It would then follow that ingestion of Immulina® by individuals may modify immune function in a manner similar to that seen with foods rich in gram-positive bacteria such as fermented milk products.

Several studies in mice have suggested that the activity of NK cells can be influenced by the intestinal microflora [24,25] and human oral consumption of bacteria (probiotics) have also been shown to enhance NK cell activity [26,27]. In general, these and other clinical trials indicate that consumption of supplements or foods containing gram-positive bacteria (bacteria in yoghurt) can result in a significant enhancement of cellular immunity indicated by the enhancement of phagocytosis by polymorphonuclear cells (PMN) and/or mononuclear cells and enhanced tumor cell killing by NK cells [28]. For example, a three-stage before and after intervention trial showed that consumption of *Bifidobacterium lactis* HN019 [29] or *Lactobacillus rhamnosus* HN001 [30] for three weeks by healthy volunteers significantly enhanced PMN phagocytosis and NK cell tumor cell killing activity. In another study, consumption of *B. lactis* HN019 by healthy elderly volunteers for three weeks enhanced PMN and mononuclear phagocytosis and NK cell activity [27]. These immune effects resulting from probiotic bacteria consumption may contribute to enhanced disease resistance. In a double-blind, placebo-controlled study with 326 children (3–5 years old), consumption of *L. acidophilus* and *B. animalis* over a six-month period resulted in statistically significant reductions over placebo in the incidence of fever (73%), coughing (62%) and rhinorrhea (59%). Antibiotic use was also reduced by 84% [31].

A recent mouse study by Akao et al. investigated the role of NK cells in the suppression of implant tumor growth by components of *Spirulina* [32]. They showed that an orally administered hot water extract of *Spirulina* reduced tumor growth and that NK cells were required for this antitumor effect. In addition, the enhancement of NK cell activity by orally administered extract was dependent on the MyD88 adaptor protein. This adaptor protein

mediates signal transduction from TLR and the results of their additional *in vitro* experiments suggested that components of the *Spirulina* extract promoted dendritic cell maturation via a MyD88/TLR2/4-dependent process and that factors produced by these dendritic cells enhanced NK cell activity. These results are consistent with our finding that Braun-type lipoproteins represent a principal active component of *Spirulina* and Immulina® and that these lipoproteins target innate immune cells through TLR2.

The mechanisms underlying the Immulina®-mediated increase in the activity of circulating NK cells remain unclear. It deserves to be investigated whether ingestion of Immulina® has an effect on the production of IL-15 by mononuclear phagocytes. IL-15 promotes NK cell development in the bone marrow [33, 34] and, like IL-2, signals through the IL-2/IL-15 beta chain (CD122) and the common gamma chain (CD132). We have previously shown that administration of Immulina® for 3 days led to an increased capability of circulating PBMC to respond to challenge with *Candida albicans* with IL-2 production [10], and it is possible that an effect of Immulina® on IL-15 and/or IL-2 production are at least partly responsible for the effects on NK cells observed in this study.

The results presented here, together with our previous studies with mice and humans, indicate that short-term, oral consumption of Immulina® can impact both gut mucosal and systemic immune functions and suggest that it may be useful for enhancing innate immune function for the prevention of infections in elderly and in other immune-suppressed individuals, or for use as a complementary therapy supporting optimal NK cell activity in cancer patients.

Acknowledgements

The authors would like to thank Ms. Annette Ford, Medical Technologist for her help in blood collection, and statistician Ms. Birgitte Rønn for her valuable contribution to the statistical treatment of our results. This research was partly funded by a USDA, Agricultural Research Service Specific Cooperative Agreement No. 58–6408–012 and a grant from Nordic Immotech A/S. Immulina® capsules were supplied by Nordic Immotech A/S, Denmark.

Affiliations

[1] Institute for Inflammation Research, Rigshospitalet National University Hospital, Copenhagen, Denmark
[2] National Center for Natural Products Research, University of Mississippi, University, MS, USA
[3] Research Institute of Pharmaceutical Sciences, University of Mississippi, University, MS, USA
[4] Department of Pharmacognosy, University of Mississippi, University, MS, USA
[5] Department of Psychology, University of Mississippi, University, MS, USA
[6] Department of Physiology, University of Mississippi, University, MS, USA
[7] Department of Cardiology P, Copenhagen University Hospital in Gentofte, Hellerup, Denmark
[8] Nordic Immotech A/S, Greve, Denmark

References

1 *Ciferri O. Spirulina*, the edible microorganism. Microbiol Rev 1983; 47: 551–578
2 *Kay RA.* Microalgae as food and supplement. Crit Rev Food Sci Nutr 1991; 30: 555–573
3 *Qureshi MA, Garlich JD, Kidd MT.* Dietary *Spirulina platensis* enhances humoral and cell-mediated immune functions in chickens. Immunopharmacol Immunotoxicol 1996; 18: 465–476
4 *Al-Batshan HA, Al-Mufarrej SI, Al-Homaidan AA, Qureshi MA.* Enhancement of chicken macrophage phagocytic function and nitrite production by dietary *Spirulina platensis*. Immunopharmacol Immunotoxicol 2001; 23: 281–289
5 *Zhang HQ, Lin AP, Sun Y, Deng YM.* Chemo- and radio-protective effects of polysaccharide of *Spirulina platensis* on hemopoietic system of mice and dogs. Acta Pharmacol Sin 2001; 22: 1121–1124
6 *Hayashi O, Hirahashi T, Katoh T, Miyajima H, Hirano T, Okuwaki Y.* Class specific influence of dietary *Spirulina platensis* on antibody production in mice. J Nutr Sci Vitaminol (Tokyo) 1998; 44: 841–851
7 *Pugh N, Ross SA, ElSohly HN, ElSohly MA, Pasco DS.* Isolation of three new polysaccharide preparations with potent immunostimulatory activity from *Spirulina platensis, Aphanizomenon flos-aquae* and *Chlorella pyrenoidosa*. Planta Med 2001; 67: 737–742
8 *Grzanna R, Polotsky A, Phan PV, Pugh N, Pasco D, Frondoza CG.* Immolina, a high-molecular-weight polysaccharide fraction of *Spirulina*, enhances chemokine expression in human monocytic THP-1 cells. J Altern Complement Med 2006; 12: 429–435
9 *Balachandran P, Pugh ND, Ma G, Pasco DS.* Toll-like receptor 2-dependent activation of monocytes by *Spirulina* polysaccharide and its immune enhancing action in mice. Int Immunopharmacol 2006; 6: 1808–1814
10 *Løbner M, Walsted A, Larsen R, Bendtzen K, Nielsen CH.* Enhancement of human adaptive immune responses by administration of a high-molecular-weight polysaccharide extract from the cyanobacterium *Arthrospira platensis*. J Med Food 2008; 11: 313–322
11 *Herberman RB, Ortaldo JR.* Natural killer cells: their roles in defenses against disease. Science 1981; 214: 24–30
12 *Talmadge JE, Meyers KM, Prieur DJ, Starkey JR.* Role of NK cells in tumour growth and metastasis in beige mice. Nature 1980; 284: 622–624
13 *Hirahashi T, Matsumoto M, Hazeki K, Saeki Y, Ui M, Seya T.* Activation of the human innate immune system by *Spirulina*: augmentation of interferon production and NK cytotoxicity by oral administration of hot water extract of *Spirulina platensis*. Int Immunopharmacol 2002; 2: 423–434
14 *Pasco DS, Pugh NDC.* Immunostimulatory composition comprising lipoprotein in microalgae extract. U.S. Patent Application Number 12/440552; 2010
15 *Eagle RA, Trowsdale J.* Promiscuity and the single receptor: NKG2D. Nat Rev Immunol 2007; 7: 737–744
16 *Kelly-Rogers J, Madrigal-Estebas L, O'Connor T, Doherty DG.* Activation-induced expression of CD56 by T cells is associated with a reprogramming of cytolytic activity and cytokine secretion profile *in vitro*. Hum Immunol 2006; 67: 863–873
17 *Chávez-Galán L, Arenas-Del Angel MC, Zenteno E, Chávez R, Lascurain R.* Cell death mechanisms induced by cytotoxic lymphocytes. Cell Mol Immunol 2009; 6: 15–25
18 *Cooper MA, Fehniger TA, Caligiuri MA.* The biology of human natural killer-cell subsets. Trends Immunol 2001; 22: 633–640
19 *Aliprantis AO, Weiss DS, Radolf JD, Zychlinsky A.* Release of Toll-like receptor-2-activating bacterial lipoproteins in *Shigella flexneri* culture supernatants. Infect Immun 2001; 69: 6248–6255
20 *Hashimoto M, Asai Y, Ogawa T.* Separation and structural analysis of lipoprotein in a lipopolysaccharide preparation from *Porphyromonas gingivalis*. Int Immunol 2004; 16: 1431–1437
21 *Hashimoto M, Tawaratsumida K, Kariya H, Aoyama K, Tamura T, Suda Y.* Lipoprotein is a predominant Toll-like receptor 2 ligand in *Staphylococcus aureus* cell wall components. Int Immunol 2006; 18: 355–362
22 *Wu HC.* Biosynthesis of lipoproteins. In: Neidhardt FC, Curtiss R, Ingraham JL, Lin ECC, Low KB, Magasanik B, Reznikoff WS, Riley M, Schaechter M, Umbarger HE, editors. *Escherichia coli* and *Salmonella*: cellular and molecular biology, 2nd ed. Washington DC: ASM Press; 1996: 1005–1014
23 *Muhlradt PF, Meyer H, Jansen R.* Identification of *S*-(2,3-Dihydroxypropyl) cysteine in a macrophage-activating lipopeptide from *Mycoplasma fermentans.* Biochemistry 1996; 35: 7781–7786
24 *Bartizal KF, Salkowski C, Balish E.* The influence of a gastrointestinal microflora on natural killer cell activity. J Reticuloendothel Soc 1983; 33: 381–390
25 *Bartizal KF, Salkowski C, Pleasants JR, Balish E.* The effect of microbial flora, diet, and age on the tumoricidal activity of natural killer cells. J Leukoc Biol 1984; 36: 739–750
26 *Takeda K, Okumura K.* Effects of a fermented milk drink containing *Lactobacillus casei* strain Shirota on the human NK-cell activity. J Nutr 2007; 137: 791S–793S

27 *Gill HS, Rutherfurd KJ, Cross ML, Gopal PK.* Enhancement of immunity in the elderly by dietary supplementation with the probiotic *Bifidobacterium lactis* HN0191. Am J Clin Nutr 2001; 74: 833–839
28 *Ezendam J, van Loveren H.* Probiotics: immunomodulation and evaluation of safety and efficacy. Nutr Rev 2006; 64: 1–14
29 *Chiang BL, Sheih YH, Wang LH, Liao CK, Gill HS.* Enhancing immunity by dietary consumption of a probiotic lactic acid bacterium (*Bifidobacterium lactis* HN019): optimization and definition of cellular immune responses. Eur J Clin Nutr 2000; 54: 849–855
30 *Sheih YH, Chiang BL, Wang LH, Liao CK, Gill HS.* Systemic immunity-enhancing effects in healthy subjects following dietary consumption of the lactic acid bacterium *Lactobacillus rhamnosus* HN001. J Am Coll Nutr 2001; 20: 149–156
31 *Leyer GJ, Li S, Mubasher ME, Reifer C, Ouwehand AC.* Probiotic effects on cold and influenza-like symptom incidence and duration in children. Pediatrics 2009; 124: e172–e179
32 *Akao Y, Ebihara T, Masuda H, Saeki Y, Akazawa T, Hazeki K, Hazeki O, Matsumoto M, Seya T.* Enhancement of antitumor natural killer cell activation by orally administered *Spirulina* extract in mice. Cancer Sci 2009; 100: 1494–1501
33 *Mrozek E, Anderson P, Caligiuri MA.* Role of interleukin-15 in the development of human $CD56^+$ natural killer cells from $CD34^+$ hematopoietic progenitor cells. Blood 1996; 87: 2632–2640
34 *Yu H, Fehniger TA, Fuchshuber P, Thiel KS, Vivier E, Carson WE, Caligiuri MA.* Flt3 ligand promotes the generation of a distinct $CD34^+$ human natural killer cell progenitor that responds to interleukin-15. Blood 1998; 92: 3647–3657