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

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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 = 0.0003 and p = 0.02, respectively). The ratio between CD56bright and CD56dim 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.

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 v9.1.3 (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, 1802–1808 Nielsen CH et al. Enhancement of Natural... Planta Med 2010; 76:
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 15) 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, γδ 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.
Approximately 10% of NK cells are known to express high levels of CD56 and are commonly referred to as CD56bright NK cells [18]. Unlike the more preponderant CD56dim NK-cell subset, the CD56bright 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 CD56dim NK cells, but are more efficient cytokine producers. We did not observe any influence of Immulina® consumption on the proportion of CD56bright and CD56dim 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-Spirulina* is a prokaryotic cyanobacteria and since Braun-Spirulina platensis* is a prokaryotic cyanobacteria and since Braun-Spirulina platensis is a prokaryotic cyanobacteria and since Braun-Spirulina platensis is a prokaryotic cyanobacteria and since Braun-Spirulina platensis is a prokaryotic cyanobacteria...
The majority of activity detected in the Immulina® extract is due to Braunt-type lipoproteins. 

**Fig. 4**

For the representative experiment that was repeated three times. **a** Immulina® extract was dissolved in 1% octylglycoside 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-Ticine 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% octylglycoside 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-dihydroxypropylcytisteine, 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 methane-sulfonic acid. Hydrolysates were analyzed using a Hewlett Packard Amino Acid Analyzer. 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-phthaldialdehyde. Peak A (2,3-dihydroxypropylcytisteine) 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 Aka 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 Brauf-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, on the production of IL-15 by mononuclear phagocytes. IL-15 enhances chemokine expression in human monocyctic THP-1 cells. [35, 36]

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.

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