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Ascophyllum nodosum extract modulates stem cell and immune cell surveillance in an acute placebo-controlled cross-over trial: Implications for healthy aging

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ABSTRACT

Background: Aging is accompanied by chronic, low-grade inflammation and immune dysfunction, highlighting a need for preventative interventions that boost the body's immune protection and innate repair mechanisms. Bioactive phytochemicals from functional foods, produced using clean, standardized technologies, are increasingly recognized and in need of scientific validation of effects in humans upon consumption.

Objective: This study evaluated acute effects of a single serving of a brown seaweed extract, PolySea, from sustainable organic wild-harvested seaweed *Ascophyllum nodosum* on immune activation, anti-inflammatory regulation, and stem cell surveillance in healthy adults. The extract was isolated by green chemistry and had a high content of polyphenols, fucoidan, and the beta-glucan laminarin.

Methods: In a randomized, double-blind, placebo-controlled, cross-over trial, twenty participants in good health consumed 50 mg of PolySea, 300 mg of PolySea, or placebo on three separate visits with at least one week's wash-out period between visits. Blood draws performed at baseline, 1 hour, and 2 hours post-consumption were analyzed for serum cytokines and changes to immune cell numbers and stem cell numbers.

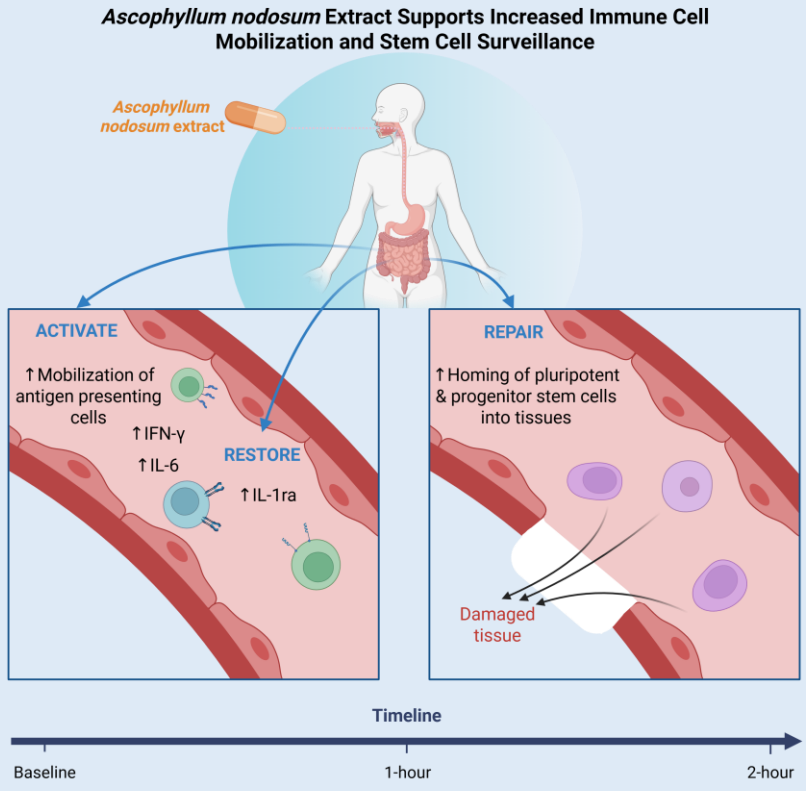
Results: Consuming a single serving of PolySea rapidly activated innate immune functions at 1 hour, where pro-inflammatory cytokines (e.g., interferon-γ, interleukin-6) were significantly elevated compared to the levels after consuming placebo. Similar effects on cytokine levels were seen for the 50 mg dose and the 300 mg dose. Concurrently, increased levels of the interleukin-1 receptor antagonist suggested anti-inflammatory restoration. One hour after

consuming 50 mg PolySea, there was a mild (4%), transient, and statistically significant increase in the number of monocytes when compared to changes after consuming placebo. A similar increase in the number of non-NK non-T cells was seen at one hour reaching a statistical trend, and at 2 hours, reaching significance. One hour after consuming 50 mg PolySea, the numbers of $CD31^{++}CD34^{-}$ endothelial stem cells were significantly higher in the blood circulation, whereas by 2 hours, $CD45^{dim}CD34^{+}$ pluripotent and progenitor stem cell numbers were significantly reduced, suggesting relocation into tissues.

Novelty of the study: The study's novelty lies in quantifying the speed and extent of acute physiological and immunological shifts induced by a single 50 mg serving of the natural seaweed extract within hours, effects that are distinct from and exceed normal circadian dynamics confirmed during the placebo visit.

Conclusion: A single serving of PolySea triggered a coordinated immune and regenerative response by rapidly triggering immune cell surveillance and selective changes to serum cytokine levels, restoring immune balance through anti-inflammatory activity, and selectively affecting different types of stem cells engaged in tissue repair. This biologically integrated response suggests meaningful potential for supporting regenerative physiological functions relevant for healthy aging.

Keywords: Anti-inflammatory; cytokine; fucoidan; immune modulation; laminarin; phlorotannin; polyphenol; senescence.



Graphical abstract: *Ascophyllum nodosum* extract supports increased immune cell mobilization and stem cell surveillance.

INTRODUCTION

At a global level, the population is aging at an unprecedented pace, with adults over 60 projected to reach 2.1 billion by 2050 [1]. As chronic disease and frailty become more prevalent, there is a growing need to extend not just lifespan but healthspan—the years lived in good health. Alongside advanced therapeutics, preventive self-care strategies are gaining attention, particularly through functional food components [2], which offer potential anti-aging benefits by enhancing the body's resilience to age-related stressors. By supporting innate defense and repair mechanisms such as immune surveillance and tissue regeneration, these natural interventions may help counteract degenerative aging processes and improve quality of life [3].

Aging can be viewed as a progressive imbalance between damage accumulation and the body's capacity for repair, ultimately leading to chronic disease and death [4]. A characteristic of this process is inflammaging, i.e., chronic, low-grade, sterile inflammation marked by elevated levels of IL-6, IL-8, and TNF-α from increasingly dysfunctional immune and nonimmune cells. While acute inflammation is essential for pathogen clearance and tissue repair, persistent inflammation contributes to tissue damage and age-related diseases.

In a typical inflammatory response, innate cells recognize pathogen-associated molecular patterns (PAMPs) from invading pathogens or damage-associated molecular patterns from dead or dying cells via their pattern-recognition receptors [5], where activation triggers signaling cascades that culminate in the production of pro-inflammatory signaling molecules like cytokines, chemokines, and growth factors that in turn stimulate recruitment of immune effectors like neutrophils and monocytes/macrophages to the site of insult. Cytokines also drive macrophage M1 polarization to clear the cellular debris or pathogen [6]. Activated antigen presenting cells process and present antigen on major histocompatibility complex (MHC) class molecules to T cells, thus linking adaptive immunity [7].

Following the pro-inflammatory phase, there is a shift to a pro-repair or anti-inflammatory wave of immune activation dominated by cellular and molecular factors that promote stem cell differentiation and tissue remodeling [8]. A shift in the cytokine milieu drives polarization of macrophages into the M2 phenotype which supports repair and remodeling through the production of cytokines and growth factors. It is an effective repair response; thus, it requires proper activation of pro-inflammatory and reparative waves of immune cell activation, timely transition between waves, and resolution once injury is eliminated [8].

The exact mechanisms behind the elevated inflammation during aging are unclear. Evidence suggests a combination of factors contribute to this phenomenon including: age-related alteration in gut microbiota composition [9-10], decline in cellular elimination systems like autophagy [11-12], and increased prevalence of senescent cells throughout the body secreting a range of inflammatory cytokines, chemokines, growth factors and matrix metalloproteinases [13]. While cellular senescence is a physiological response to numerous cellular stressors and serves important roles like preventing the spread of damaged cells [14], the senescence-associated secretory phenotype (SASP) contributes to producing an inflammatory environment and can induce senescence in neighboring cells [15]. Immune cells and stem cells cooperate to limit this accumulation of senescent cells. SASP factors and stress-induced ligands recruit and activate immune cells that recognize and clear senescent cells [16]. SASP factors stimulate stem cell proliferation and differentiation to replace old and damaged cells [17-19].

With aging, the mechanisms to limit senescent cell burden fail as the innate and adaptive immune responses become increasingly dysfunctional and stem cells

become less responsive [20]. Additionally, the stem cells and immune cells themselves become susceptible to chronic inflammation [21-22]. SASP factors can interfere with stem and progenitor cell function, including driving myeloid-biased expansion, metabolic reprogramming, and niche remodeling [23-24]. Proliferation of stem cells in response to pro-inflammatory signals can lead to accumulation of DNA damage which in turn can trigger apoptosis and senescence [17]. Inflammatory compounds secreted by aging cells further increase immune cell dysfunction [25].

Given the relationship between stem cell dysfunction, immunosenescence, and premature aging, strategies to enhance the gut-immune-stem cell axis are a promising therapeutic target. Current approaches include small molecule senolytics, which aim to limit senescent cells largely by targeting senescent cell anti-apoptotic pathways, senomorphics, which modulate SASP, immune-based therapies, and stem cell-based therapies. Issues with these approaches include a lack of universal senescence markers, a reliance on animal models, possible off-target effects, and senescent cell heterogeneity [26]. Further research is needed for possible synergistic effects of combinatorial approaches that target both the immune and stem cell axes with validation of these approaches in human clinical trials.

Among natural sources of bioactive compounds with potential health benefits, seaweed has gained increasing attention for their rich content of molecules such as fucoidan, laminarin, and polyphenols. Fucoidan, a negatively charged, sulfated polysaccharide, composed of L-fructose [27], found in the extracellular matrix of several algal species, has demonstrated a considerable list of desirable effects, ranging from antioxidant to anticancer [28, 29]. Fucoidan is readily absorbed via endocytosis, found in vital organs after oral consumption [30] and in both the serum and urine of people who regularly consume seaweed as a food source [31]. The anti-cancer effects of fucoidan are achieved through various mechanisms. Injection of fucoidan inhibits mitosis, arresting the cell cycle [32]. Fucoidan can also induce apoptosis in cancer cells by activating caspases [33], causing the inhibition of formation of VEGF [34]. Fucoidan has been shown to be able to mobilize hematopoietic stem cells from bone marrow to the peripheral blood [35]. Fucoidan has also been shown to have immune modulatory effects including inducing dendritic cell maturation, enhancing adaptive responsiveness, and interacting with toll-like receptors to stimulate immune responses [36]. Fucoidan also has anti-inflammatory effects through its inhibitory effects on selectins, the complement system [37, 38], and on enzymes like elastases and matrix metalloproteases [39]. We have previously discussed the role of L-selectins in stem cell mobilization using an algae-based extract enriched for an L-selectin ligand [40].

Laminarin is another polysaccharide found in seaweed that produces effects similar to fucoidan upon consumption. Laminarin demonstrates antioxidant activity in vitro, scavenging free radicals [41, 42] and can induce apoptosis via the activation of caspases [43]. Laminarin is also source of dietary fiber and has demonstrated the ability to influence gut microbiota, producing a shift towards carbohydrate digestion [44]. In zebrafish, in addition to bolstering antioxidant activity, laminarin was able to promote fin regeneration via improved adhesion and migration of cells [45-46].

Phlorotannins are polyphenols found in brown seaweed that provide structure for algal cell walls, promote wound healing, and provide UV protection [47]. Phlorotannins, like laminarin and fucoidan, exhibit potent antioxidant activities such as scavenging free radicals and inhibiting intracellular reactive oxygen species generation [48-50]. Phlorotannins can also cross the blood brain barrier, allowing them to induce a neuroprotective effect through various mechanisms; they inhibit the formation of plaques, as well as reduce the activation of NF-kB and MAPK pathways in microglial

cells [51-52]. In epithelial cells, phlorotannin has similar anti-inflammatory effects preventing the release of LPS-induced TNF-α and IL-6 [53]. Phlorotannins have antimicrobial activity, inhibiting the growth of methicillin-resistant *Staphylococcus aureus* with synergistic effects when used with β-lactams [54]. Phlorotannins increase mitochondrial activation, promoting ATP production while inhibiting ROS, and they upregulate the Nrf1 pathway which includes key regulators of mitochondrial biosynthesis and function [55-56].

The North Atlantic brown seaweed *Ascophyllum nodosum* contains considerable levels of fucoidan, laminarin, and phlorotannins among other bioactive compounds. *A. nodosum* is widely utilized in agriculture, cosmetics, and nutritional supplements with an extensive history of in vitro and in vivo studies [57]. Medical treatments involving stem cell techniques often necessitate injections, high costs, or donor cells [58], while some algae-based natural products can improve stem cell characteristics [35] and enhance surveillance via consumption [40]. Such a preventative measure is minimally invasive and thus acceptable to healthy individuals, as well as individuals with health conditions.

The objective of the clinical trial presented here was to document the acute effects of the novel brown seaweed polyphenol-rich extract PolySea compared to placebo. Healthy subjects were tested using an established placebo-controlled, randomized, double-blinded, cross-over study design [59], in which each participant acted as their own control [39, 60-61]. The timing of blood draws on the days when 50 mg versus 300 mg of PolySea was consumed was matched to the timing on the day placebo was consumed, of importance for controlling each participant's normal circadian changes [62]. This study focused on changes in serum cytokine levels, immune cell surveillance, and stem cell surveillance.

METHODS

Study Design: This clinical trial was conducted according to a randomized double-blinded placebo-controlled cross-over study design (clinical trial registration NCT07142720) which was conducted in accordance with the Declaration of Helsinki, and approved by the Argus Independent Review Board, Tucson, AZ, USA. The cross-over study design, in which each participant was tested on both doses of active product as well as placebo, follows a similar design as previously published clinical trials [39, 60, 61], and has greater statistical power than a parallel-arm design with a larger sample size, due to the reduction in inter-subject variability, and allowing for within-subject analysis. The determination of sample size was based on previous trials on polyphenol-rich and β-glucan-rich nutritional supplements [60-61]. The in/exclusion profile and recruiting process was performed as described previously [59], where the exclusion criteria were predefined, uniformly applied, and based solely on safety and methodological considerations, thereby minimizing the potential for selection bias. We aimed for a minimum of 16 people to complete the study and achieved results from all 20 enrolled. Data from all 20 people (Table 1) were included in the analysis (Figure 1).

Table 1. Demographics of the study population.

	N	Age average[1]	Age range	BMI average[1]	BMI Range
Females	11	63.1 ± 6.3	55.6 – 75.8	26.6 ± 4.2	20.6 – 34.3
Males	9	51.1 ± 21	22.6 – 75.2	25.4 ± 3.4	19.6 – 29.2

[1] The average ± standard deviation is shown.

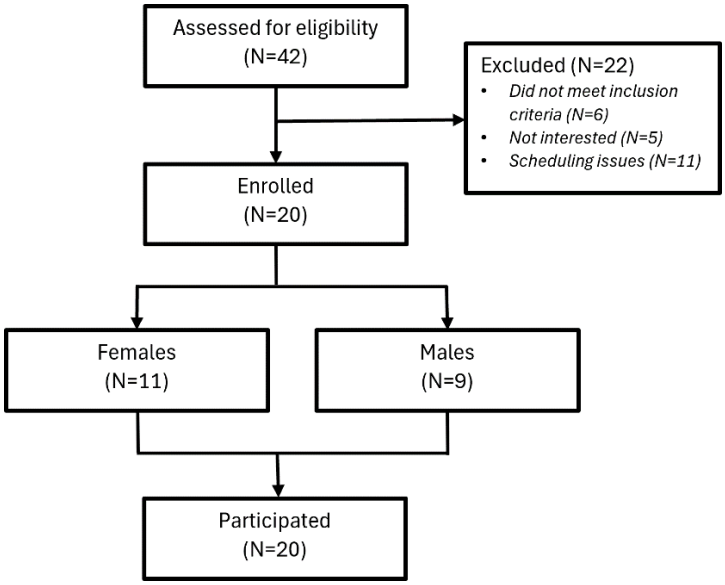


Figure 1. CONSORT flow chart showing the number of people that were assessed for eligibility, excluded, enrolled, and participated.

Study participants attended three clinic visits separated by at least 7 days wash-out period (Figure 2). The participants received a single serving of placebo and the active product 1 week apart. Below is a diagram illustrating the involvement of each participant.

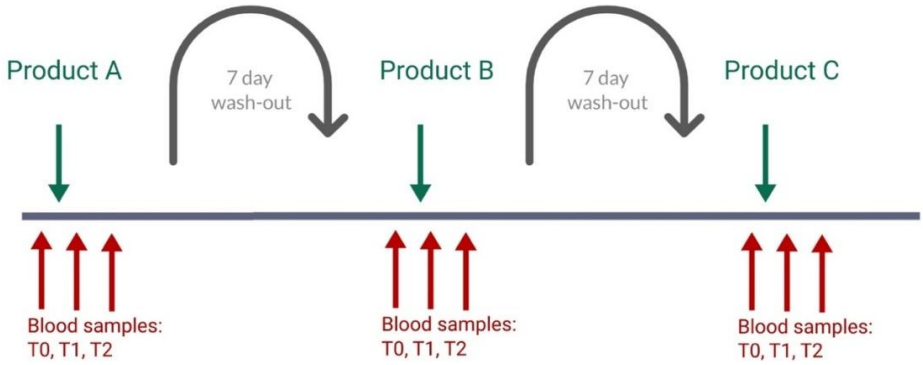


Figure 2. Diagram displaying the schedule for each study participant. The order in which participants received 50 mg PolySea, 300 mg PolySea, or placebo was randomized.

Each participant was scheduled to attend visits consistently in mornings between 7 and 11 AM to control for circadian fluctuations of the biomarkers relevant to this trial for all three visits where 50 mg PolySea, 300 mg PolySea, and placebo were consumed. Due to the known effects of stress and exercise on these biomarkers, the clinical environment was managed to reduce stress. As each person arrived for their appointments, a brief survey ensured that no unexpected stress, sleep issues, or health issues were affecting the person's ability to complete the appointment, following preset criteria for whether rescheduling was needed. A mandatory 1-hour period of rest was followed by the baseline blood draw, which was followed by consuming a test product. A small bland snack was served to stimulate gastric function. For the baseline, 1-hour and 2-hour blood draws, one serum separator tube and one heparin tube was drawn. The blood in the serum separator tube coagulated for 30–60 minutes, was spun, and serum was transferred to a conical tube and spun cold. Serum was aliquoted and

banked at −80°C until later cytokine testing. Immune and stem cell staining were performed using the heparinized blood and was started within an hour of each blood draw.

Consumable test product: The brown seaweed-based *Ascophyllum nodosum* extract, PolySea™, was provided as a powder from SeaChange Biochemistry, Dartmouth, Nova Scotia, Canada. Proximate analysis was conducted at Colombia Labs (Portland, Oregon, USA) to determine the overall composition of the sample. The analysis showed that the sample contained 12.3% moisture, 0.90% total fat, 4.91% protein, and 23.7% ash. From these values, total carbohydrates were calculated by difference as 58.2 g/100 g, with total sugars at 0.26 g/100 g, resulting in a combined organic fraction of 58.46 g/100 g.

To determine the identity and relative composition of major organic constituents in PolySea, quantitative 1H nuclear magnetic resonance (NMR) spectroscopy was performed at the Nuclear Magnetic Resonance Research Resource, Dalhousie University (Halifax, Nova Scotia, Canada). NMR samples were prepared by dissolving 19 mg of PolySea in 0.75 mL of D_2O. Data were acquired on a 500 MHz Bruker Neo spectrometer using the noesygppr1d pulse sequence with 128 scans and 8 dummy scans. This experiment collected a proton spectrum with presaturation water suppression. The relaxation delay was fixed at 3 seconds. Raw NMR data were processed with a 0.5 Hz exponential line-broadening function prior to Fourier transformation. Transformed spectra were phase-corrected, and a baseline-flattening routine was applied before analysis. NMR analysis identified fucoidan, mannitol, polyphenols, and laminarin as the predominant analytes. On a dry-weight (dw) basis, the concentrations were: Fucoidan: 25.9% dw, Mannitol: 21.0% dw, Polyphenols: 12.6% dw, and Laminarin: 7.3% dw. Together, these four compounds accounted for nearly 67% of the dry extract, indicating that the material was highly enriched in bioactive polysaccharides and phenolic compounds.

When preparing PolySea for consumption during the clinical trial, serving sizes of 50 mg powder and 300 mg powder were dissolved in 45 mL plain rice milk to camouflage the seaweed taste. The placebo consisted of 45 mL plain rice milk.

Reagents: Phosphate-buffered saline (PBS), High Yield Lyse™ and Cal-Lyse™ whole-blood lysing solutions; and monoclonal antibodies: CD69 (FITC), CD56 (PE), CD3 (SB645), CD31 (FITC), CD90 (SB436) and CD45 (Pacific Orange) were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Serum separator tubes (SST), heparin vacutainer tubes; 21G butterfly blood collection needles, and monoclonal antibodies: CD309 (VEGFR-2), CD34 (PerCP) and CD25 (BV421) were purchased from Becton-Dickinson (Franklin Lakes, NJ, USA). Bio-Plex Pro™ human cytokine arrays were purchased from Bio-Rad Laboratories Inc. (Hercules, CA, USA).

Flow Cytometry Evaluation of Immune Cell Numbers and Phenotype: Whole blood was used to evaluate changes in immune cell numbers and phenotype, where each blood sample was tested in triplicate, as described previously [60, 61]. Analysis was performed using the Attune software, where electronic gates were set on forward/side scatter to identify monocyte and lymphocyte populations. The lymphocytes were then gated based on CD3 and CD56 markers, to allow documentation of changes to the numbers and CD25/CD69 expression on $CD3^+CD56^-$ T cells, $CD3^+CD56^+$ NKT cells, $CD3^-CD56^+$ NK cells, and $CD3^-CD56^-$ non-NK non-T cells.

Flow Cytometry Evaluation of Stem Cells: Whole blood was used to evaluate changes to the numbers of different

types of stem cells, where each blood sample was tested in triplicate, as described previously [59]. Staining was performed by a "no wash" procedure involving Cal-Lyse® Lysing solution fixation of white blood cells and lysing of red blood cells. For each triplicate sample, 300,000-600,000 events were collected. Analysis was performed to document changes to the numbers of stem cells per microliter of sample, compensating for the dilution in the "no wash" protocol, so that the data were transformed to stem cell numbers per microliter blood. This allowed analysis of changes in stem cell numbers within $CD45^{dim}CD34^{+}$ classical stem cells divided into two subsets $CD45^{dim}CD34^{+}CD309^{+}$ pluripotential stem cells and $CD45^{dim}CD34^{+}CD309^{-}$ progenitor cells.

Cytokines, chemokines, and growth factors: From each blood sample, serum was tested for levels of 27 cytokines and chemokines using the Bio-Plex Pro Human Cytokine 27-plex magnetic bead arrays (cat# M500KCAF0Y, Bio-Rad Laboratories Inc.) [60]. A MagPix microplate reader was used to record and analyze the results using xPonent software (Version 4.2, Luminex, Austin, TX, USA).

Data Analysis: Averages and standard errors of the mean were calculated in Microsoft® Excel® for Microsoft 365 (Microsoft Corporation, Redmond, WA, USA, version 2507). Baseline values were compared between clinic visits for each data set, and did not show significant changes, indicative of the wash-out being sufficient, and no carry-over effects detected. Changes from baseline were calculated at each time point using simple arithmetic calculations of the arithmetic mean ± standard error of the mean for between-treatment comparison. Changes were summarized as mean ± standard error of the mean to characterize responses after consuming a 50 mg dose versus a 300 mg dose of PolySea relative to placebo. Differences between changes were calculated by subtracting changes happening when placebo was consumed. This allowed evaluation of product-specific changes [59, 62]. Both the changes and the differences in changes were evaluated using within-subject analysis of changes from baseline, as described previously [59]. Analyses were conducted using available data only, with participants excluded from specific analyses when relevant measurements were missing, and no imputation was performed. Within-subject variability was handled by analyzing each time point separately, avoiding inappropriate pooling of repeated measurements. The levels of statistical significances were defined as $p<0.1$ (trend), $p<0.05$ (significant), and $p<0.01$ (highly significant) using conventional benchmarks. However, given the exploratory nature and size of the dataset, we have also emphasized effect sizes and consistency of observed trends. Findings with potential clinical relevance are discussed even when statistical significance was not reached.

RESULTS AND DISCUSSION

Changes to Immune Activating Cytokines: Consumption of PolySea triggered highly selective changes in the serum cytokine levels (Figure 3). While both the 50 mg and the 300 mg doses influenced IL-6 at 1 hour, there were also noticeable differences: For the lower 50 mg dose, seven cytokines showed changes, where three reached statistical significance (IFN-γ, IL-6, and IL-1ra) and four reached statistical trends (IP-10, RANTES, IL-4, and GM-CSF). For the higher 300 mg dose, two of the seven cytokines reached statistical significance (IL-6, PDGF-BB) and five reached statistical trends (IL-12(p70), IL-13, eotaxin, RANTES, and G-CSF).

At one hour after consumption, comparable magnitudes of effects were seen for most cytokines for both the 50 mg serving size and the 300 mg serving size (Figure 3), with IL-6 reaching statistical significance for both doses, and IFN-γ and IL-1ra reaching statistical significance for the lower dose.

Thirteen cytokines were detectable in serum samples from all 20 participants at all time points. Due to a lab error, data from some cytokines from one participant for the 1-hour blood draw on the day where placebo was consumed were removed from analysis.

Not all cytokines were detectable in all participants, and data from a participant were only analyzed for detectable cytokines. As an example, data for IFN-γ were removed from three participants where this cytokine was not detectable.

The heat map for the magnitude (Figure 3, right panel) shows a biphasic effect for the 50 mg dose for IL-8 and TNF-α, but less so for the 300 mg dose. Notably, the 50 mg dose triggered reduced levels of the inflammatory cytokine IL-1β (P<0.25), and while not reaching statistical significance, this may reflect a biologically relevant anti-inflammatory effect. Based on the clearer response characteristics of the lower dose, subsequent data presentation focuses on the 50 mg dose compared to placebo (Figures 4-7).

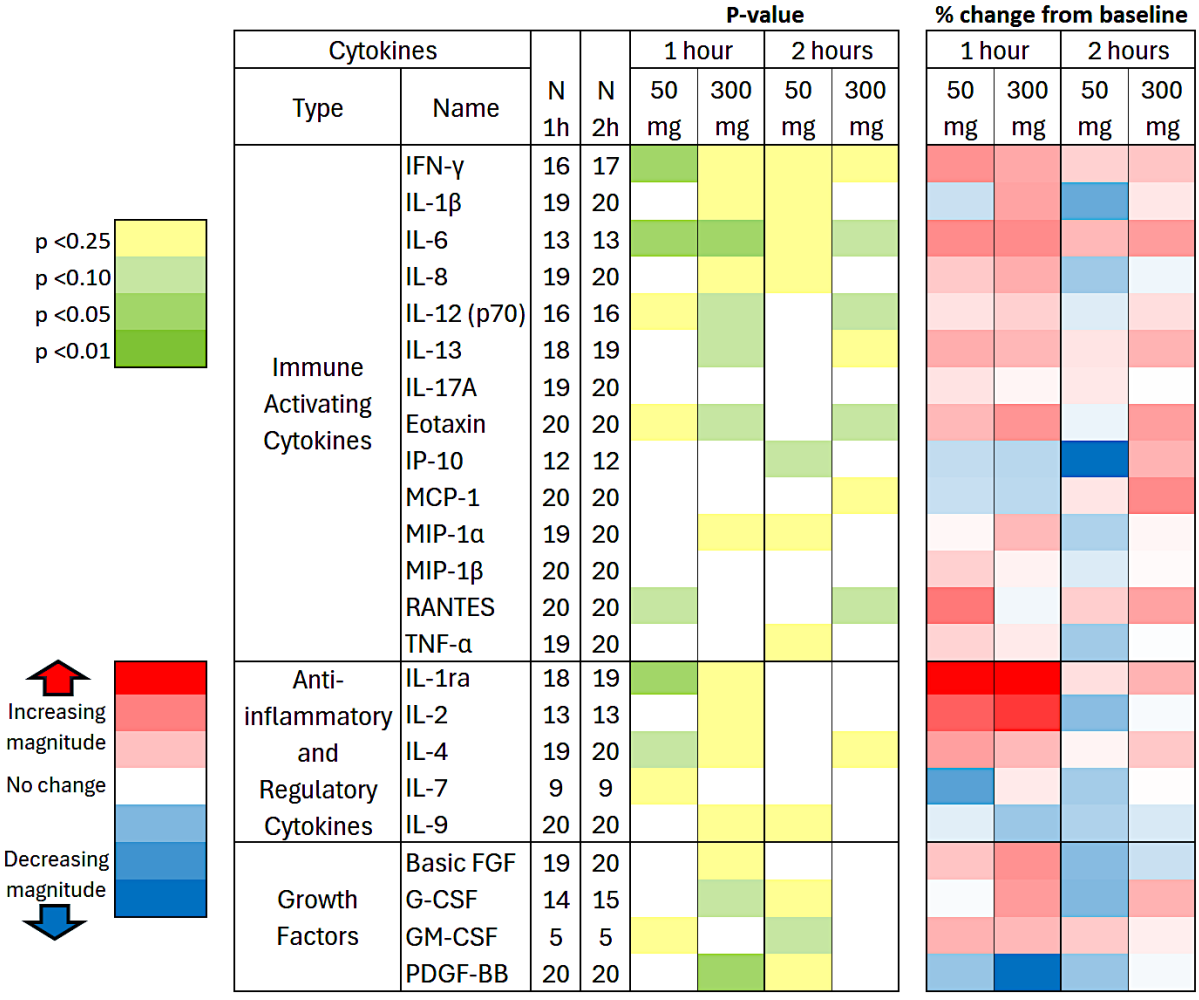


Figure 3. Effects of PolySea consumption on serum cytokine levels: Density maps showing changes from baseline 1 and 2 hours after consumption of 50 mg versus 300 mg PolySea when compared to changes after consuming placebo. The left heat map displays levels of statistical significance, and the right heat map displays the relative magnitudes of the increases versus decreases to cytokine levels.

After consuming 50 mg PolySea, the immune activating cytokines IFN-γ, IL-6, and RANTES showed increased levels compared to changes after consuming the placebo after 1 and 2 hours (Figure 4). IFN-γ (Figure 4B) and IL-6 (Figure 4D) showed rapid increases at 1 hour after consuming PolySea when compared to placebo, where the difference in changes reached statistical significance ($p < 0.05$). RANTES levels also rapidly

increased, where the difference between changes after consuming PolySea versus placebo reached a statistical trend at 1-hour post-consumption (P<0.1) (Figure 4H). In contrast, IP-10 levels were reduced after consuming PolySea, where the difference in changes after consuming PolySea versus placebo reached a statistical trend at 2 hours (p<0.1) (Figure 4F). It was important to note that the levels of other pro-inflammatory cytokines were down-regulated even if they did not reach statistical significance, including IL-1β, IL-8, MCP-1, MIP-1α, MIP-1β, and TNF-α.

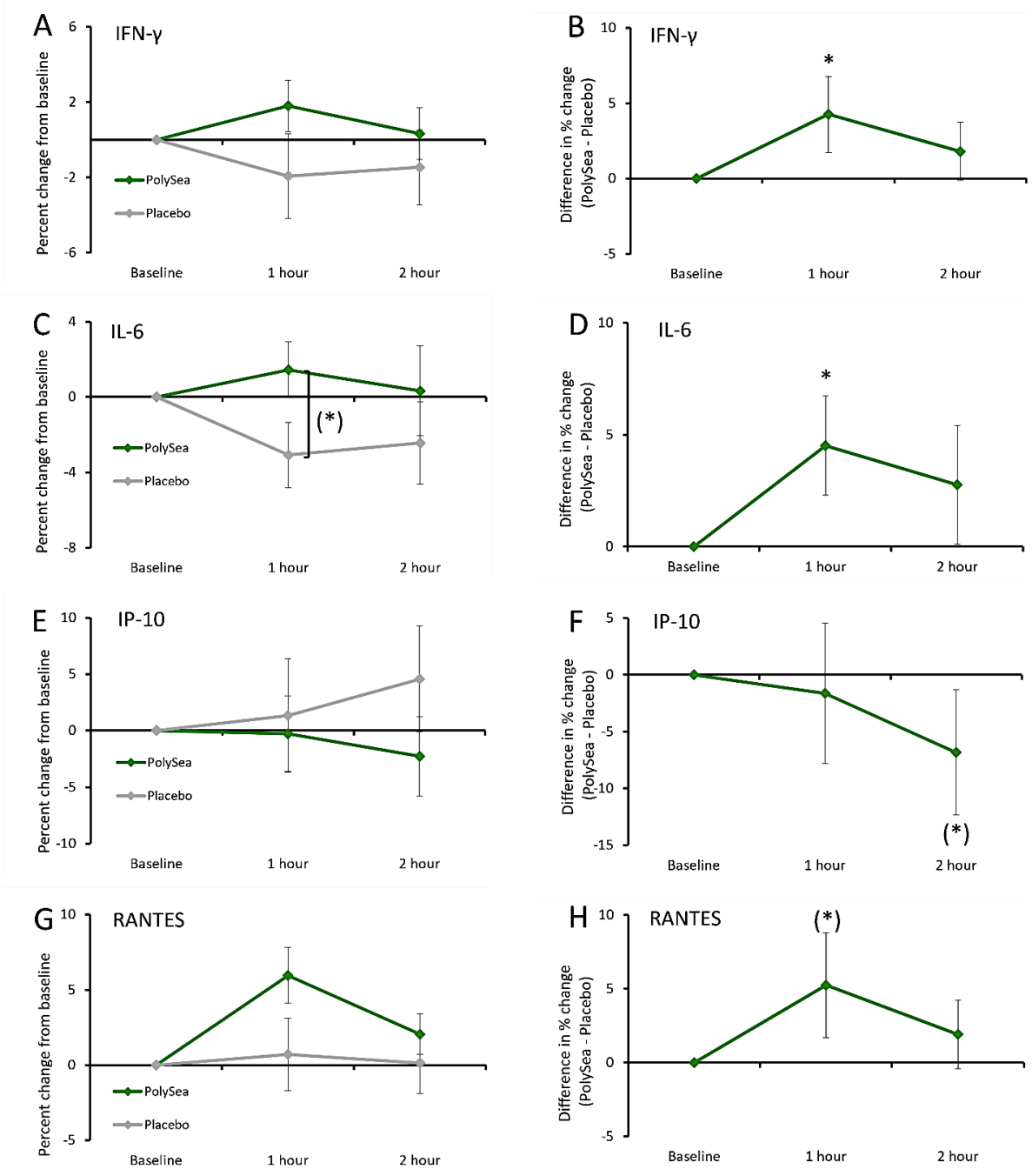


Figure 4. Effects of 50 mg PolySea on serum pro-inflammatory cytokines. Left panels show the changes after consuming PolySea (green) compared to changes after consuming placebo (grey). Right panels show placebo-adjusted changes. Averages ± SEM are shown for 1 hour and 2 hours after consumption. Levels of significance: p<0.10: (*) and p<0.05: *.

Changes to Anti-inflammatory Cytokines: Rapid and selective changes to anti-inflammatory cytokine levels were also seen after consuming PolySea compared to changes after consuming placebo (Figure 5). IL-1ra levels (Figure 5A, B) increased 1 hour after consuming PolySea, where the difference between changes after consuming PolySea versus placebo reached statistical significance ($p<0.05$) (Figure 5B). Serum levels of IL-4 (Figure 5C, D), a cytokine with anti-inflammatory and immunoregulatory functions, also increased 1 hour, where the difference between changes after consuming PolySea versus placebo reached a statistical trend ($p <0.1$) (Figure 5D).

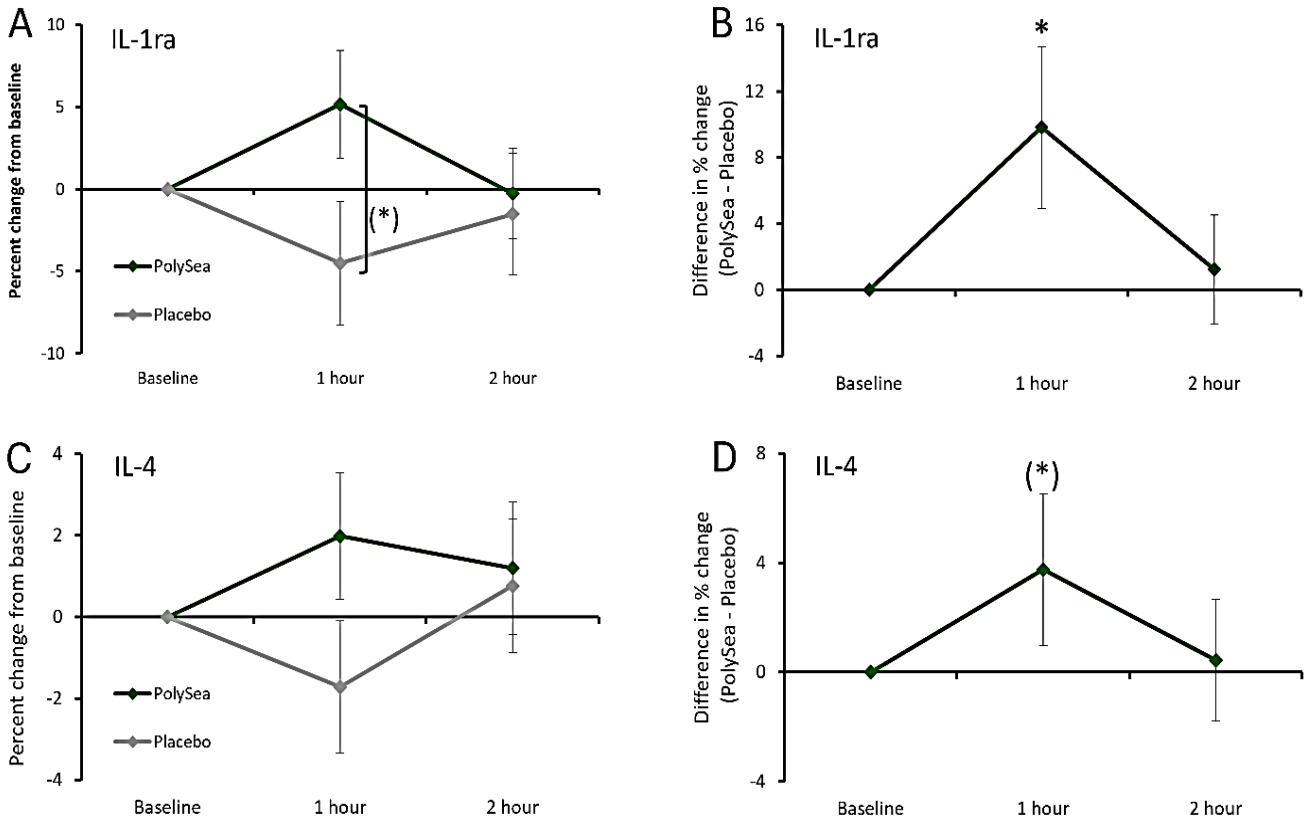


Figure 5. Effects of 50 mg PolySea on serum anti-inflammatory cytokines. Left panels show the changes after consuming PolySea (green) compared to changes after consuming placebo (grey). Right panels show placebo-adjusted changes. Averages ± SEM are shown for 1 hour and 2 hours after consumption. Levels of significance: $p<0.10$: (*), $p<0.05$: *, and $p<0.01$: **.

Immune Cell Surveillance: Consuming 50 mg PolySea was associated with rapid changes to the numbers of specific immune cell types in blood circulation, compared to the changes observed after placebo was consumed (Figure 6). At 1 hour after consuming PolySea, the number of monocytes increased compared to changes after consuming placebo, where the difference between changes after consuming PolySea versus placebo reached statistical significance ($p <0.05$, Figure 6B). An increase in non-NK non-T cells was seen at both 1 and 2 hours after consuming PolySea, where the difference between changes after consuming PolySea versus placebo reached a statistical trend at 1 hour ($p <0.1$) and statistical significance at 2 hours ($p<0.05$, Figure 6H). In contrast, consumption of PolySea did not significantly affect the numbers of NK cells (Figure 6C, D) or T cells (Figure 6E, F) at either 1 hour or 2 hours post consumption compared to placebo.

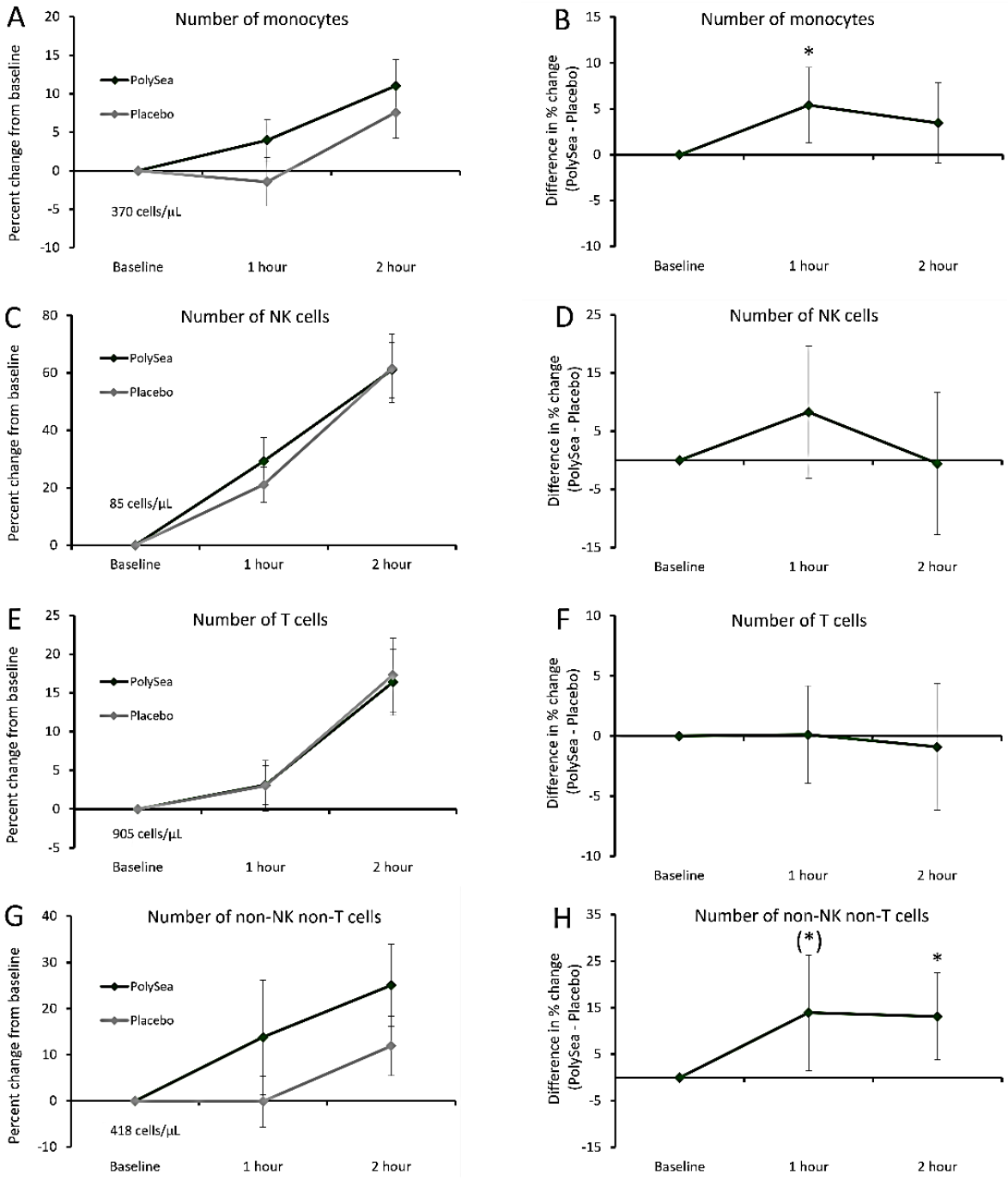


Figure 6. Effects of 50 mg PolySea on immune cell surveillance. Left panels show the changes in cell numbers after consuming PolySea (green) compared to changes after consuming placebo (grey), where the average cell numbers at baseline are indicated for each cell type. Right panels show the placebo-adjusted changes. Averages ± SEM are shown for 1 hour and 2 hours after consumption. Levels of statistical significance: p<0.10: (*), p<0.05: *, and p<0.01: **.

Stem Cell Surveillance: Consuming a single 50 mg dose of PolySea was associated with changes to the numbers of several types of circulating stem cells (Figure 7). These changes included both subtypes of the $CD45^{dim}CD34^{+}$ stem cell population, based on whether the stem cells expressed CD309 (vascular endothelial growth factor-receptor 2 (VEGFR-2), the kinase insert domain receptor (KDR)). At 2 hours after consuming PolySea, there was a decrease in number of $CD45^{dim}CD34^{+}CD309^{+}$ pluripotent stem cells compared to placebo (Figure 7A), where the

difference between changes after consuming PolySea versus placebo reached statistical significance ($p<0.05$, Figure 7B). Similarly, at 2 hours after consuming PolySea, there was a decrease in the number of $CD45^{dim}CD34^{+}CD309^{-}$ progenitor stem cells (Figure 7C), where the difference between changes after consuming PolySea versus placebo reached statistical significance ($p<0.05$, Figure 7D). In contrast, consuming PolySea triggered an increase in the number of $CD31^{++}CD34^{-}$ endothelial stem cells at 1-hour post-consumption (Figure 7E), where the difference between changes after consuming PolySea versus placebo was significant ($p<0.05$, Figure 7F). The number of $CD31^{++}CD34^{-}$ endothelial stem cells remained elevated at 2 hours post-consumption (not significant) (Figure 7E, F).

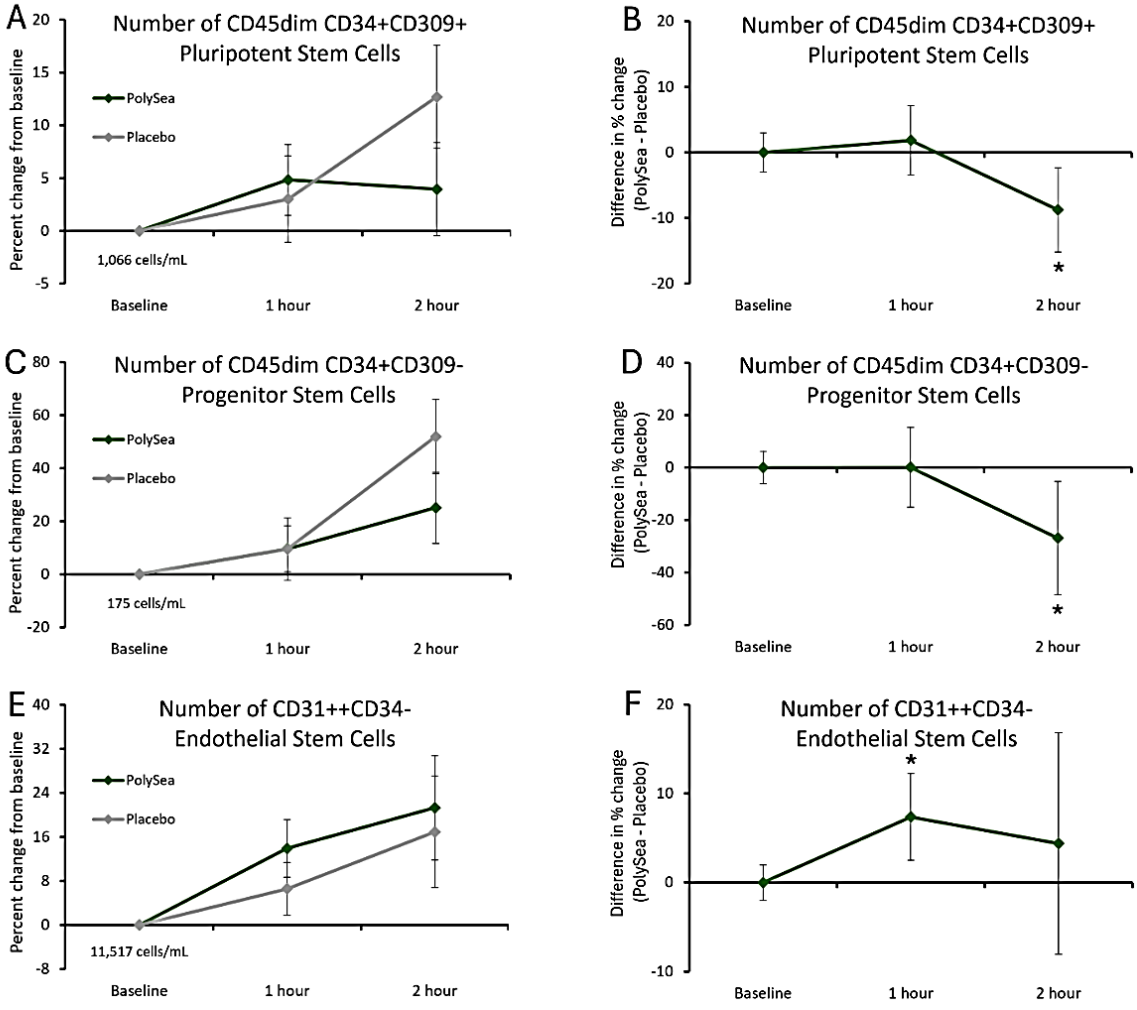


Figure 7. Effects of 50 mg PolySea on stem cell surveillance. Left panels show the changes after consuming PolySea (green) compared to changes after consuming placebo (grey), where the average cell numbers at baseline are indicated for each cell type. Right panels show the placebo-adjusted changes. Averages ± SEM are shown for 1 hour and 2 hours after consumption. Levels of statistical significance: $p<0.05$: *.

Discussion: The goal for the clinical trial presented herein was to compare the acute effects of consumption of a single serving of the *Ascophyllum nodosum* extract PolySea to placebo through the evaluation of serum cytokine levels, immune cell surveillance, and stem cell surveillance. Two doses of PolySea were compared: 50 mg and 300 mg, and two timepoints: 1 and 2 hours, were used for evaluation of post-consumption effects. The study is aligned with the Functional Food Center's development steps related to dose, timing, and relevant biomarkers for clinical efficacy [62], controlling for individual differences in normal circadian changes,

relevant for the study of personalized nutrition strategies. The PolySea extract is rich in the bioactive compounds fucoidan, laminarin, and phlorotannins, and supported rapid and selective changes in serum cytokine levels, immune cell mobilization, and stem cell homing into tissues, properties that go beyond basic nutrition.

Consuming a single serving of either 50 mg or 300 mg PolySea was associated with highly selective changes in serum cytokine levels within 1 hour after consumption. These changes were characterized by increases in the immune activating cytokine IL-6 and the anti-inflammatory cytokine IL-1ra for both doses, reaching statistical significance for both cytokines for the 50 mg dose. For the lower 50 mg dose, increased levels of IFN-γ also reached statistical significance at 1 hour. The lower dose triggered milder changes to RANTES and IP-10, increasing and decreasing respectively. Notably, reduced levels of other pro-inflammatory cytokines including IL-1β, IL-8, IL-17, MCP-1, MIP-1α, MIP-1β and TNF-α were seen but did not reach significance, suggesting that PolySea triggered a tailored activation of the immune response, distinctively different from a broad systemic inflammatory reaction. By the same 1-hour timepoint, there was a significantly increased level of the anti-inflammatory cytokine IL-1ra for the 50 mg dose. Only the low dose showed a significant increase in IFN-γ and IL-1ra. Both the low and high dose showed significant increased levels of IL-6 at 1 hour. This immune-activating pro-inflammatory effect remained a statistical trend for the high dose at 2 hours, suggesting a slower resolution and return to homeostasis after consuming the higher dose, especially considering the less significant increase in the anti-inflammatory cytokine IL-1ra after consuming the higher dose. The results suggest similar activating properties by both doses, with the lower dose triggering a more beneficial cytokine profile with a faster resolution. This suggests that consuming the 50 mg dose of PolySea had an immunomodulatory effect, stimulating immune cells but also harnessing the response through anti-inflammatory activity.

Based on the comparable magnitudes of changes after healthy adults consumed both doses of PolySea but noting that the changes after consuming the 50 mg dose were more robust for key immune-activating and regulating cytokines, further data presentation focused on the lower 50 mg dose as the more efficacious in this cohort. In parallel with the cytokine changes, consumption of 50 mg PolySea was associated with rapid changes to immune cell populations. These changes were also selective, as ingestion was associated with statistically significant mobilization of monocytes and the CD56-CD3- non-NK non-T subset of lymphocytes into the bloodstream, while numbers of T cells and NK cells remained unchanged. The non-NK non-T lymphocyte subset contains antigen-presenting dendritic cells, and their increase, together with the increase in monocyte numbers, implies that PolySea consumption stimulated increased numbers of antigen-presenting innate immune cells in the blood circulation.

The observed effects of PolySea on immune surveillance may be explained mechanistically by the biological activity of the extract's laminarin and fucoidan components. Laminarin, a β-glucan with β-(1,6) and β-(1,3) glycosidic linkages, is known to engage primarily with Dectin-1 pattern recognition receptors, but also with Complement Receptor 3, and weakly with toll-like receptor (TLR) 2/4 [36]. Dectin-1 receptors are predominantly expressed on macrophages, neutrophils and dendritic cells, and ligand binding triggers NF-κB and MAPK-mediated release of cytokines including IL-6 [63]. Fucoidan, a sulfated polysaccharide, also contributes to immunostimulation by engaging TLR-2, TLR-4, and C-type lectin receptors on immune cells, which promote secretion of nitric oxide, TNF-α, IL-1β, and IL-6 and can activate NK cells, promoting IFN-γ release [64, 65]. Moreover, systemic administration of fucoidan is associated with enhanced dendritic cell maturation and function, increasing levels of co-stimulatory factors and

secretion of pro-inflammatory cytokines like IL-6 [66]. Fucoidan and laminarin can activate NF-κB by several pathways, and research has shown that laminarin and fucoidan may combine to enhance macrophage activity [67]. Further investigation in humans is warranted to understand potential synergistic effects of the combined mechanisms of action of laminarin and fucoidan on immune stimulation.

IFN-γ has several roles in cell-mediated immunity and anti-viral defense. IFN-γ suppresses viral replication in infected cells by inducing the expression of antiviral genes and enhancing the presentation of viral antigens, thereby disrupting the viral life cycle, and promoting immune-mediated clearance [68]. IFN-γ is predominantly released by activated lymphocytes and antigen presenting cells [68, 69]. Effects of IFN-γ signaling include upregulation of co-stimulatory molecules and antigen processing and presentation, orchestrating Th1 effector mechanisms, and polarization of macrophages towards an M1 “classical” pro-inflammatory functional state [70].

IL-6 has pleiotropic roles in inflammation, immune surveillance, and stem cell behavior, among other processes [71]. IL-6 is secreted in response to cell injury, damage or stress, and has context-dependent effects on inflammation, shaping the immune responses, and regulating hematopoiesis [72]. IL-6 also has a role as a myokine as it is released by contracting skeletal muscle fibers acting in a paracrine and endocrine manner to exert numerous biological effects, including stimulating muscle hypertrophy and myogenesis [73]. Further work is needed to see if this IL-6-boosting ability of PolySea may be beneficial for supporting physical activity in the aging community via the myokine effects of IL-6.

In addition to its pro-inflammatory roles, IL-6 signaling is known to promote the production of anti-inflammatory cytokines IL-1ra and IL-10 [74, 75]. IL-1ra, a member of the IL-1 cytokine family, is widely expressed and competitively antagonizes the effects of both IL-1α and IL-1β by having greater affinity for their IL-1R1 receptors [76]. The simultaneous rise of the anti-inflammatory cytokine IL-1ra following intake of PolySea suggests that PolySea may trigger anti-inflammatory pathways and may be a downstream consequence of IL-6 induction.

Consumption of 50 mg PolySea also had rapid effects on stem cell behavior. There was a rapid and transient rise in the numbers of CD31++CD34- endothelial stem cells at 1-hour post-consumption. This increase in endothelial stem cell mobilization coincided with the observed rise in IL-6, which is known to stimulate endothelial progenitor cell proliferation [77]. These cells are implicated in vascular repair and angiogenic processes [78], and their rise might suggest that consuming the extract initiated endothelial repair. Two hours after consumption, blood levels of CD34+CD309+ pluripotential stem cells and CD34+ CD309- progenitor stem cells were significantly reduced. The effects of PolySea on stem cell dynamics may be associated with the actions of fucoidan on stem cell migration and homing. Orally ingested fucoidan can be detected in blood plasma [79], peaking around 2 hours [80]. Fucoidan has been reported to interfere with stem cell retention signals in the bone marrow niche by competing with ligands for L-selectin (CD62L), thereby causing mobilization of stem cells into circulation [35, 40]. Moreover, fucoidan stimulates upregulation of CXCR4 on stem cells, which increases stem cell sensitivity to stromal cell-derived factor-1 (SDF-1, CXCL12), a chemokine that is upregulated in damaged tissues [81]. These mobilized CXCR4+ stem cells can then home to sites of damage following the CXCL12 chemotactic gradient [82]. Fucoidan may also bind SDF-1 directly, stabilizing its dimeric form and preserving a chemotactic signal for CXCR4+ stem cells at sites of injury [83]. With the effects on pluripotential and progenitor stem cell surveillance observed occurring slightly slower than the cytokine/immune changes, together with the known biology of fucoidan, our data supports a model of a coordinated response: an initial wave of immune and endothelial activation with anti-inflammatory feedback, followed by pluripotential and progenitor stem cell recruitment. Further studies that involve stem cell tracking in tissues would be valuable to confirm that the relocated pluripotential and progenitor stem cells were

in fact residing in and rejuvenating sites of tissue damage or senescence.

In summary, the results reported here are suggestive of an orchestrated timeline of events where consumption of the brown seaweed *A. nodosum* extract PolySea enhanced immune surveillance, restored homeostasis, and signaled to endothelial, pluripotential, and progenitor stem cells to migrate, which is part of normal repair or rejuvenation (Figure 8). We propose that the fucoidan and laminarin components of PolySea initiate the stimulatory effect, at the level of the gut mucosal immune tissue, as well as through systemic effects. Anti-inflammatory mechanisms provide feedback control on immune stimulation. IL-6 stimulates IL-1ra production which inhibits IL-1β signaling. Concurrently, the phlorotannin component of PolySea contributes to its anti-inflammatory activity, as it has been demonstrated to have potent antioxidant and anti-inflammatory effects, including inhibiting inflammatory signaling via NF-κB and MAPK involved in triggering the production of pro-inflammatory cytokines [84, 85]. Phlorotannins are also capable of downregulating TLR 2/4 expression, NO release, and prostaglandin production in addition to their broad antioxidant activity [84]. Phlorotannins are able to penetrate immune cells and inhibit MAPK and NF-κB, therefore limiting cytokine production and excessive inflammation. In the late phase, fucoidan competes with L-selectin ligands within bone marrow tissue, thereby freeing stem cells from docking and allowing their relocation into the blood circulation, followed by homing into tissues in need of repair. The increased homing may be related to fucoidan's ability to increase sensitivity of stem cells to SDF-1 released by damaged tissues, through upregulation of CXCR4 expression on the stem cell membrane, leading to stem cells homing to sites of damage following the chemotactic gradient [35].

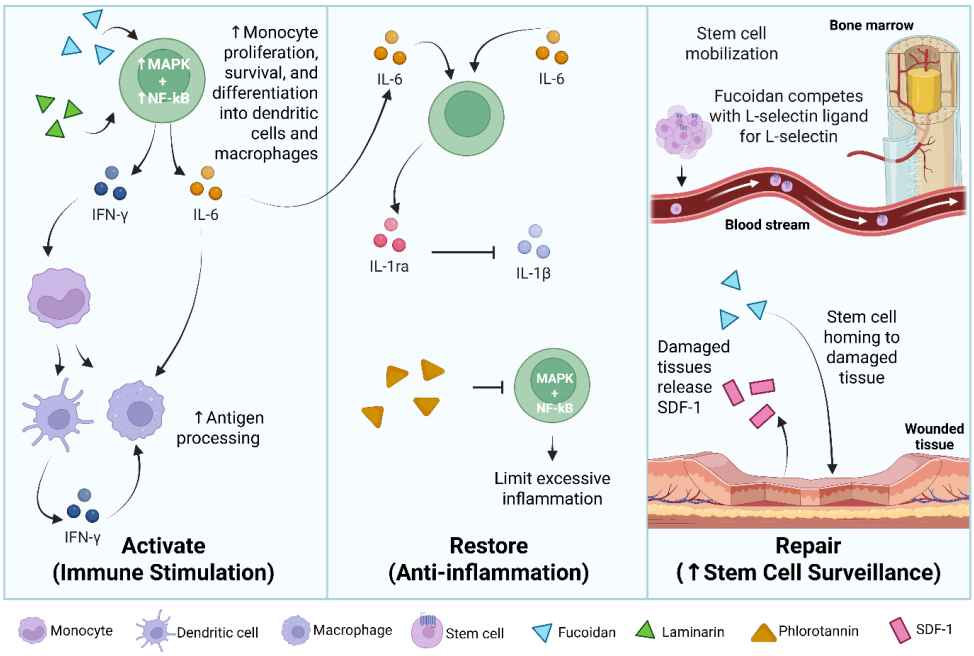


Figure 8. Schematic diagram summarizing the mechanisms of action at the cellular level for each of the major compounds in the *A. nodosum* extract PolySea. Left panel: Fucoidan engages with TLR-2/4 receptors on immune cells, while laminarin acts via the Dectin-1 receptor, resulting in secretion of interferon-gamma (IFN-γ) and interleukin-6 (IL-6), converging in MAPK and NF-kB signaling and further increasing the production of IL-6 and IFN-γ. These cytokines act on their receptors to induce effects on antigen-presenting cells. Center panel: IL-6 production also leads to increased IL-1ra production, which together with the activity of phlorotannins limits the pro-inflammatory immune stimulation. Right panel: In the late phase, fucoidan liberates stem cells from the bone marrow niche and increases their sensitivity to SDF-1, a chemokine relevant for tissue recruitment of stem cells after tissue damage.

The results of this study support a mechanistically plausible case of a natural product with anti-aging benefit and sets the stage for further investigations. The complex extract effects of PolySea were tested, but while the observed responses were tentatively linked to specific bioactive components, the precise contribution of each component within the PolySea extract needs to be elucidated. Further work may test individual active components in parallel to document the relative contribution of each component to the observed biological effects and to demonstrate the potential synergistic effect of the multifaceted extract. Follow-up studies may also expand on cytokine testing. Considering PolySea's effect on IFN-γ, it would be useful to test interferon-α and -β to determine if consuming PolySea triggers broader antiviral protection. The conclusions made on stem cell homing would benefit from further validation by in vivo fluorescent cell tracking. The dose of 50 mg in healthy individuals, optimal in this trial when compared to the 300 mg dose, may need reevaluation in different cohorts [86], where subsequent dose studies should also include evaluation of effects on the gut microbiota [87, 88]. While the study reported here included healthy people of up to 75 years of age, it would also be beneficial to tie the effects seen to functional outcomes in an elderly population by conducting a long-term study in an ageing cohort above the age of 75, stratified according to physical and mental health decline.

Aging is a multifactorial process associated with multiple interconnected domains of dysfunction including immunosenescence, chronic inflammation, and stem cell hyporesponsiveness. This dysfunction is strongly correlated with the prevalence of age-related chronic degenerative conditions and frailty. While most current therapeutic anti-aging interventions tend to target a single pathway with modest benefit, emerging evidence suggests that combining interventions that target multiple pathways can have additive or synergistic effects [89, 90]. The ability of PolySea to support immune activation, anti-inflammation, and tissue repair is highly relevant in the context of supporting healthy aging through long-term preventative strategies involving dietary bioactive compounds [91].

CONCLUSION

The clinical trial reported here has documented that consumption of a single dose of the brown seaweed *Ascophyllum nodosum* extract PolySea has selective and rapid effects on communication via cytokines, immune surveillance, and stem cell homing into tissues. The polysaccharide and polyphenol components in PolySea act in a concerted effort to directly and specifically modulate pathways related to immune activation, cytokine production, and anti-inflammatory protection. Consuming PolySea triggered systemic changes to immune status and stem cell effects involved in repair and rejuvenation. These acute effects on the gut-immune-stem cell axis suggest that long-term daily consumption of PolySea may have potential impact for preventing and reversing senescence and may be an effective nutraceutical strategy for supporting healthy aging.

List of Abbreviations: IFN-γ, Interferon-gamma; IL-1β, Interleukin-1 beta; IL-1ra, Interleukin-1 receptor antagonist; IL-4, Interleukin-4; IL-6, Interleukin-6; IL-8, Interleukin-8; IL-10, Interleukin-10; IL-17, Interleukin-17; IP-10, IFN-γ-inducible protein 10; KDR, Kinase insert domain receptor; MAPK, Mitogen-activated protein kinase; MCP-1, Monocyte chemoattractant protein-1; MIP-1α, Macrophage inflammatory protein-alpha; MIP-1β, Macrophage inflammatory protein-beta; NF-κB, Nuclear factor kappa-light-chain-enhancer of activated B cells; RANTES, Regulated on activation, normal T cell expressed and secreted; SASP, Senescence-associated secretory phenotype; SDF-1, Stromal cell-derived factor-1; TLR, Toll-like receptor; TNF-α, Tumor necrosis factor-

alpha; VEGF, Vascular endothelial growth factor; VEGFR-2, Vascular endothelial growth factor receptor 2.

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Competing interests: The authors declare no conflict of interest. The clinical trial was conducted at NIS Labs, an independent contract research lab specializing in natural products research. NIS Labs received funding from SeaChange Biochemistry, Inc., to conduct research on the seaweed extract, but neither NIS Labs nor any of the coauthors have other financial ties to the study sponsor or the nutraceutical extract studied here. The funder had no role in the design of the study; in the collection, analyses, audit, or interpretation of data; or in the writing of the manuscript. All data analysis and manuscript preparation were conducted independently by the authors. The corresponding author had full access to all the data and final responsibility for the decision to submit for publication.

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Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

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