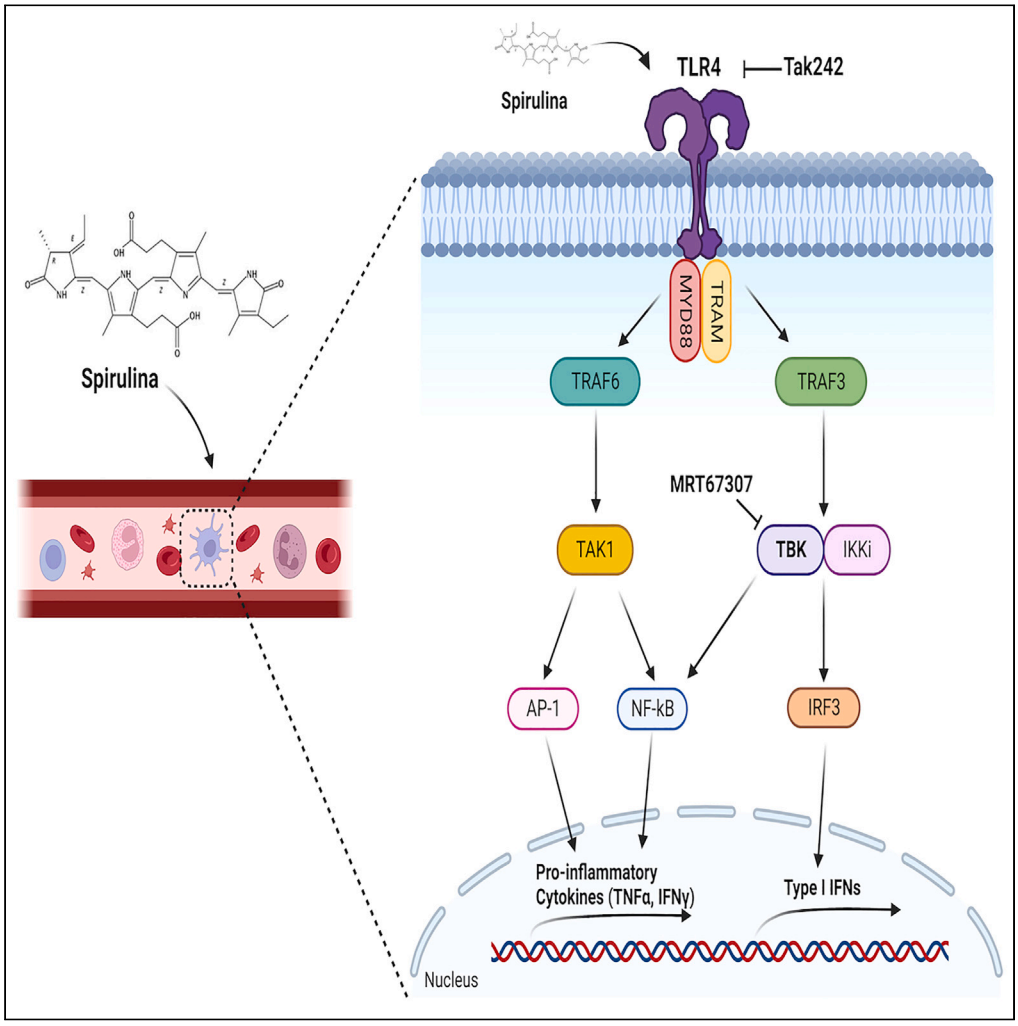
iScience

CellPress
OPEN ACCESS

Article

Herbal supplement Spirulina stimulates inflammatory cytokine production in patients with dermatomyositis *in vitro*



Christina E. Bax, DeAnna Diaz, Yubin Li, ..., Daisy Yan, Muhammad Bashir, Victoria P. Werth

werth@pennmedicine.upenn.edu

Highlights

Spirulina stimulates several inflammatory cytokines production in Dermatomyositis

Spirulina promotes autoimmunity via the production of IFNγ and TNFα in Monocytes

Spirulina's stimulation is greater in Dermatomyositis than healthy controls

Spirulina's stimulation is via the TLR4 pathway in both blood and skin

Bax et al., iScience *26*, 108355
November 17, 2023
https://doi.org/10.1016/j.isci.2023.108355

iScience

CellPress
OPEN ACCESS

Article

Herbal supplement Spirulina stimulates inflammatory cytokine production in patients with dermatomyositis *in vitro*

Christina E. Bax,[1,2,3] DeAnna Diaz,[1,2,3] Yubin Li,[1,2,3] Thomas Vazquez,[1,2] Jay Patel,[1,2] Madison Grinnell,[1,2] Adarsh Ravishankar,[1,2] Spandana Maddukuri,[1,2] Emily Keyes,[1,2] Daisy Yan,[1,2] Muhammad Bashir,[1,2] and Victoria P. Werth[1,2,4,*]

SUMMARY

Spirulina, an herbal supplement and popular ingredient in health foods, is a potent stimulant of the immune system. Spirulina use is temporally associated with the onset or exacerbation of Dermatomyositis (DM), an autoimmune connective tissue disease that frequently affects the skin and muscle. In this study, we investigated the effect of Spirulina on peripheral blood mononuclear cells (PBMCs) in DM and Healthy Controls (HCs), showing that Spirulina stimulates Interferon β (IFNβ), Tumor necrosis factor α (TNFα), and Interferon γ (IFNγ) production of DM PBMCs primarily via Toll-Like Receptor 4 (TLR4) activation using ELISA (enzyme linked immunosorbent assay) and flow cytometry. We show that classical monocytes and monocyte-derived dendritic cells are stimulated by Spirulina and are activated via TLR4. Skin from patients with Spirulina-associated DM exhibits an inflammatory milieu similar to that of idiopathic DM but with a stronger correlation of TLR4 and IFNγ.

INTRODUCTION

Spirulina platensis (Spirulina) is a blue-green algae hailed as a "super food" due to its purported health benefits.[1,2] It is widely used in tablet form or as a powder in energy bars, smoothies, and green juices.[2] Patients with dermatological diseases are increasingly using herbal supplements,[3] and among patients with autoimmune skin disease surveyed, patients with DM, in particular, frequently consume Spirulina, with 15% of patients with DM in our prior study reporting Spirulina consumption, nearly 4 times more than controls.[4] In addition to reports of onset or exacerbation of autoimmune disease following Spirulina consumption,[5–7] our recent epidemiologic data suggests that Spirulina is temporally associated with the onset or exacerbation of DM.[8,9]

Spirulina is made up of hundreds of nutrients, including proteins, fatty acids, and vitamins, as well as many different functional compounds with various immunostimulatory, anti-inflammatory, and antioxidant effects.[10] Pure Spirulina consumption, rather than specific components of Spirulina, has been studied as a potent innate immune system booster and adjuvant treatment in patients with Human Immunodeficiency Virus (HIV) and Acquired Immunodeficiency Syndrome (AIDS), with several studies demonstrating increased Clusters of Differentiation 4 (CD4) count and reduced viral load in individuals with Spirulina supplementation.[11,12] Moreover, due to its immunostimulatory effects, it has also been studied as adjuvant therapy in patients with cancer to further boost the immune system and reduce myelosuppression.[13] While Spirulina also contains phenolic antioxidants and C-phycocyanin, which have been found to exert anti-inflammatory effects via the modulation of the Nuclear factor erythroid 2-related factor 2 (Nrf2) and Nuclear factor kappa B (NF-kB) pathways,[10] numerous *in vitro* and *in vivo* studies have also shown that Spirulina stimulates the immune system,[14,15] including by activating natural killer (NK), CD4 T, CD8 T, and monocyte cells.[16–18] Isolated components of Spirulina increase many inflammatory cytokines including Interleukin 8 (IL-8), Monocyte chemoattractant protein 1 (MCP-1), Macrophage inflammatory protein 1-alpha (MIP-1α), Macrophage inflammatory protein 1-beta (MIP-1β), Interferon gamma inducible protein (IP-10), TNFα, Interleukin 1-beta (IL-1β), the enzyme Cyclo-oxygenase-2 (COX-2), Interleukin 4 (IL-4), and IFNγ.[18–20] As toll-like receptors are one of the main activators of the innate immune system, ultimately leading to the activation of the NF-κB pathway and downstream inflammatory cytokines, we sought to further explore Spirulina's effects on TLR activation.[21]

DM is a rare autoimmune connective tissue disease characterized by muscle, skin, and/or lung involvement. Inflammatory cytokines including IFNβ, IFNγ, and TNFα are upregulated in patients with DM and have been implicated in disease pathogenesis.[22–25] Pathways of

[1]Corporal Michael J. Crescenz VA Medical Center, Philadelphia, PA, USA
[2]Department of Dermatology, University of Pennsylvania, Philadelphia, PA, USA
[3]These authors contributed equally
[4]Lead contact
*Correspondence: werth@pennmedicine.upenn.edu
https://doi.org/10.1016/j.isci.2023.108355

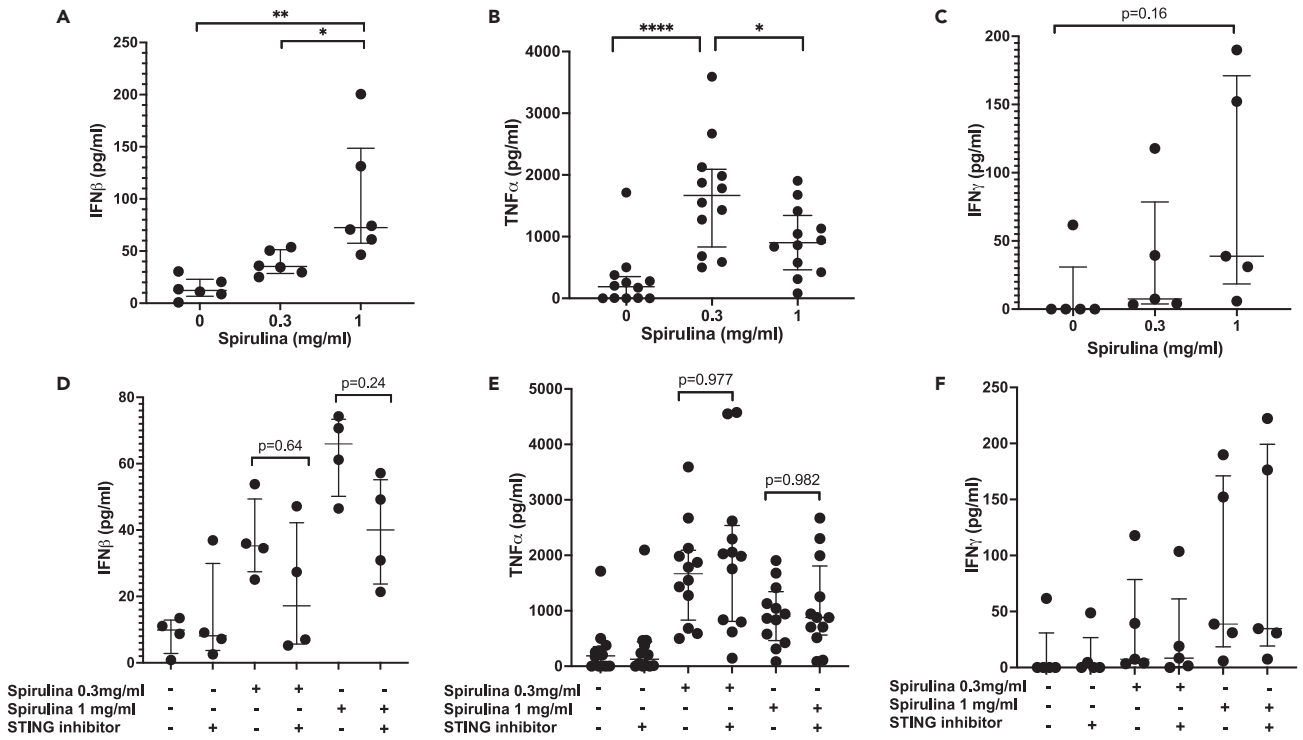


Figure 1. Herbal supplement Spirulina stimulates the secretion of inflammatory cytokines IFNβ, TNFα, and IFNγ in DM PBMCs by ELISA

(A) Quantification of IFNβ production by PBMCs stimulated with 0, 0.3, or 1 mg/mL of Spirulina (n = 6).

(B) Quantification of TNFα production by PBMCs stimulated with 0, 0.3, 1 mg/mL of Spirulina by Enzyme linked immunosorbent assay (n = 12).

(C) Quantification of IFNγ production by PBMCs stimulated with 0, 0.3, 1 mg/mL of Spirulina (n = 5).

(D–F) Quantification of IFNβ, TNFα and IFNγ production by PBMCs stimulated with 0.3 or 1 mg/mL of Spirulina in the presence of STING inhibitor. Bars show *p < 0.05, **p < 0.01, ****p < 0.0001. Lines represent the median and interquartile range.

interest in DM include TLR4 activation[26] and the Stimulator of Interferon Genes (STING) pathway, which is increasingly being recognized as an important pathway in autoimmunity, leading to increased Type 1 interferon.[27–29]

To our knowledge, the role that Spirulina may play in inducing or exacerbating autoimmune skin diseases and upregulating inflammatory pathways has never been studied. Moreover, monocyte subsets that Spirulina may activate and cellular-level differences between autoimmune and healthy control responses to Spirulina have not previously been examined.

The purposes of this study are to 1) investigate the immunostimulatory effects of the popular herbal supplement Spirulina *in vitro*; 2) elucidate the pathways that Spirulina activates in PBMCs of patients with DM; 3) compare the immune profile of lesional DM skin in patients with Spirulina-associated DM; and 4) identify which cells Spirulina activates *in vitro* in DM PBMCs to contribute to inflammatory cytokine production.

RESULTS

Spirulina stimulates the secretion of inflammatory cytokines interferon β, tumor necrosis factor α, and interferon γ in dermatomyositis peripheral blood mononuclear cells

The effect of Spirulina on inflammatory cytokine release for all three cytokines was evaluated. Supernatant from Spirulina-treated DM PBMCs contained IFNβ 14.2 ± 4.2, 38.3 ± 4.7 pg/mL, and 97.4 ± 23.8 pg/mL at 0, 0.3, and 1 mg/mL Spirulina (mean ± SEM, n = 6), respectively. Spirulina significantly increased IFNβ secretion from PBMCs at 1 mg/mL compared to non-stimulated cells ($p < 0.01$) and dose-dependently increased IFNβ secretion from 0.3 mg/mL to 1 mg/mL ($p < 0.05$) (Figure 1A).

TNFα secretion also increased, peaking at 0.3 mg/mL Spirulina stimulation. Supernatant from stimulated DM PBMCs contained TNFα 292.7 ± 138.0 pg/mL, 1671.7 ± 258.1 pg/mL, and 933.9 ± 158.0 pg/mL at baseline and at 0.3 and 1 mg/mL Spirulina concentrations, respectively (mean ± SEM, n = 12). Spirulina significantly increased supernatant TNFα levels from DM PBMCs at 0.3 mg/mL compared to non-stimulated cells ($p < 0.0001$) (Figure 1B). 10 μg/mL lipopolysaccharide (LPS) was tested next as a positive control (n = 4), with immunostimulatory effects on TNFα production in DM PBMCs comparable to Spirulina 0.3 mg/mL ($p = 0.897$) (Figure S1).

Spirulina stimulation increased IFNγ production from DM PBMCs. Supernatant from PBMCs treated with Spirulina contained IFNγ 12.3 ± 12.3, 34.4 ± 21.9, and 83.6 ± 36.6 pg/mL at 0, 0.3 and 1 mg/mL Spirulina concentration, respectively (mean ± SEM, n = 5), although the effect was variable between patients with DM ($p = 0.16$) (Figure 1C).

Similar effects were seen with respect to the immunostimulatory effects of Spirulina on TNFα and IFNγ production in healthy control (HC) PBMCs, with significant increases in cytokine production at 0.3 mg/mL and 1 mg/mL Spirulina stimulation compared to baseline (Figures S2A and S2B).

Inhibition of stimulator of interferon genes pathway does not attenuate interferon β, tumor necrosis factor α, or interferon γ production from Spirulina-stimulated peripheral blood mononuclear cells in patients with dermatomyositis

Pre-treatment with 1μM STING inhibitor H-151 prior to Spirulina stimulation did not significantly affect the secretion of IFNβ, although levels trended downwards in all four patients tested. Spirulina 0.3 mg/mL stimulated IFNβ to 37.3 ± 6.0 pg/mL, with a decrease to 21.7 ± 9.9 pg/mL with STING inhibitor ($p = 0.64$). Spirulina 1 mg/mL stimulated IFNβ to 63.2 ± 6.2 pg/mL, with a decrease to 39.5 ± 8.2 pg/mL with STING inhibitor ($p = 0.24$) (Figure 1D).

STING inhibition had no effect on TNFα production at 0.3 mg/mL Spirulina ($p = 0.91$), with mean level (SEM) of 1671.7 ± 258.1 pg/mL to 2021.6 ± 406.4 pg/mL nor at 1 mg/mL, with mean level 933.9 pg/mL ± 158.0 to 1086.6 ± 238.7 pg/mL ($p > 0.99$) ($n = 12$) (Figure 1E). Similarly, STING inhibition did not attenuate IFNγ production by Spirulina-stimulated DM PBMCs at 0.3 mg/mL ($p > 0.99$) and 1 mg/mL ($p > 0.99$) ($n = 5$) (Figure 1F).

Similar effects were seen when evaluating the effects of STING inhibitor on TNFα and IFNγ production in HC Spirulina-stimulated PBMCs (Figures S2C and S2D).

Both TANK-Binding Kinase-1 (TBK1) and Toll-like receptor 4 inhibition significantly decrease tumor necrosis factor α and interferon γ production in Spirulina-stimulated peripheral blood mononuclear cells in patients with dermatomyositis and healthy controls

Other possible pathways contributing to upregulated inflammatory cytokines were next tested by evaluating the effects of TLR4 and TBK1 inhibition on TNFα and IFNγ production in a cohort of DM PBMCs treated with 0.3 mg/mL Spirulina, which is closest to estimated physiological blood levels after oral Spirulina consumption (discussed in supplemental methods).

Pre-treatment with 5μM TBK1 inhibitor significantly suppressed the secretion of TNFα by Spirulina-stimulated PBMCs in patients with DM, with supernatant levels decreasing from 1838.8 ± 344.8 pg/mL at 0.3 mg/mL Spirulina stimulation to 356.0 ± 150.9 pg/mL with TBK1 inhibition ($p < 0.0001$) (mean ± SEM, $n = 4$) (Figure 2A). Similarly, pre-treatment with 1 μg/mL TLR4 inhibitor significantly decreased TNFα to 132.6 pg/mL ± 89.8 ($p < 0.0001$) ($n = 4$) (Figure 2A). Similar results were seen in HC PBMCs, with supernatants of Spirulina-stimulated DM PBMCs decreasing from 1706.0 pg/mL ± 271.4 with 0.3 mg/mL Spirulina stimulation to 276.6 ± 107.4 pg/mL with TBK1 inhibition ($p = 0.0001$) and to 269.3 ± 141.2 pg/mL with TLR4 inhibition (mean ± SEM, $n = 5$) ($p = 0.0001$) (Figure 2B). There was no difference in the percent decrease in TNFα levels between Spirulina-stimulated HC PBMCs and DM PBMCs in the presence of TLR4 and TBK1 inhibitors (Tukey's multiple comparisons test, $p = 0.96$ and $p = 0.99$, respectively).

For IFNγ, pre-treatment of DM PBMCs with TBK1 inhibitor or TLR4 inhibitor prior to 0.3 mg/mL Spirulina stimulation decreased IFNγ levels (Figure 2C), although the effect was not statistically significant. Among HC PBMCs, Spirulina-stimulated IFNγ levels significantly decreased with the pre-treatment of TBK1 inhibitor ($p < 0.001$) or TLR4 inhibitor ($p < 0.001$) (Figure 2D).

Spirulina increases activity of interferon β, tumor necrosis factor α, and interferon γ by $CD14^{++}CD16$ classical monocytes and $CD11c^{+}CD14^{+}$ monocyte-derived dendritic cells in dermatomyositis peripheral blood mononuclear cells. this effect is significantly greater than that seen in healthy controls

We next utilized flow cytometry to investigate the effects of Spirulina at the cellular level. We determined which cell lineages were activated by Spirulina, leading to increased levels of IFNβ, TNFα, and IFNγ in the supernatant. A second question was whether Spirulina activates patients with DM and HCs differently, which may help elucidate why Spirulina negatively affects only some patients with DM.

Eight cell lineages [CD4 T, CD8 T, $CD11c^{+}$ classical dendritic cells (cDC), $CD123^{+}$ plasmacytoid dendritic cell (pDC), CMs, $CD14^{++}CD16^{+}$ intermediate monocytes (IMs), $CD14^{+}CD16^{++}$ non-classical monocytes (nCMs), and moDCs] were evaluated using flow cytometry. Representative gating after treatment with 0 and 0.3 mg/mL Spirulina is shown (Figure 3A).

Spirulina 0.3 mg/mL had the greatest effect on the DM IFNβ activity of CMs (mean 31.7 ± 4.7% increase from baseline) (mean ± SEM, $p < 0.05$) (Figure 3B) and upregulated DM CM activity significantly more than HC CMs ($p < 0.001$) (Figures 3B and 3C). In HCs, the percent of activated CMs was significantly lower at 0 mg/mL Spirulina than in DM (24.9 ± 8.7 vs. 58.3 ± 6.1% active, mean ± SEM, $p = 0.031$), and actually decreased with Spirulina. In addition, the percentage of IFNβ-activated CMs in HCs with mean 6.8 ± 1.2% compared to mean 89.0 ± 7.9% in DMs at 0.3 mg/mL Spirulina ($p < 0.0001$) and a difference was also seen at 1 mg/mL Spirulina ($p < 0.0001$) with mean 7.8 ± 1.7% in HC vs. 82.5 ± 9.4% in DM. (Figure 3C).

When evaluating TNFα activity from PBMCs, there was a significant increase in the percentage of activated CMs (50.1 ± 6.0%, mean ± SEM, $p < 0.0001$) and in activated moDCs (39.0 ± 10.3%, mean ± SEM, $p < 0.001$) relative to other cell lineages in DM PBMCs when stimulated with 0.3 mg/mL Spirulina compared to 0 mg/mL Spirulina (Figures 3D–3F). While the percentage of $TNFα^{+}$ CMs was similar between DMs and HCs at baseline ($p > 0.99$), Spirulina 0.3 mg/mL and 1 mg/mL stimulated significantly more $TNFα^{+}$ CMs in DM samples than in HC samples ($p < 0.0001$ and $p = 0.0005$), respectively (Figure 3E). Similarly, while there was no difference in percent of $TNFα^{+}$ moDCs between DM and HC at baseline, there were significantly more TNFα-producing moDCs in DM (43.6 ± 7.8% active) after 0.3 mg/mL Spirulina stimulation compared to HCs (mean 10.0 ± 4.3% active) ($p < 0.001$) (Figure 3F).

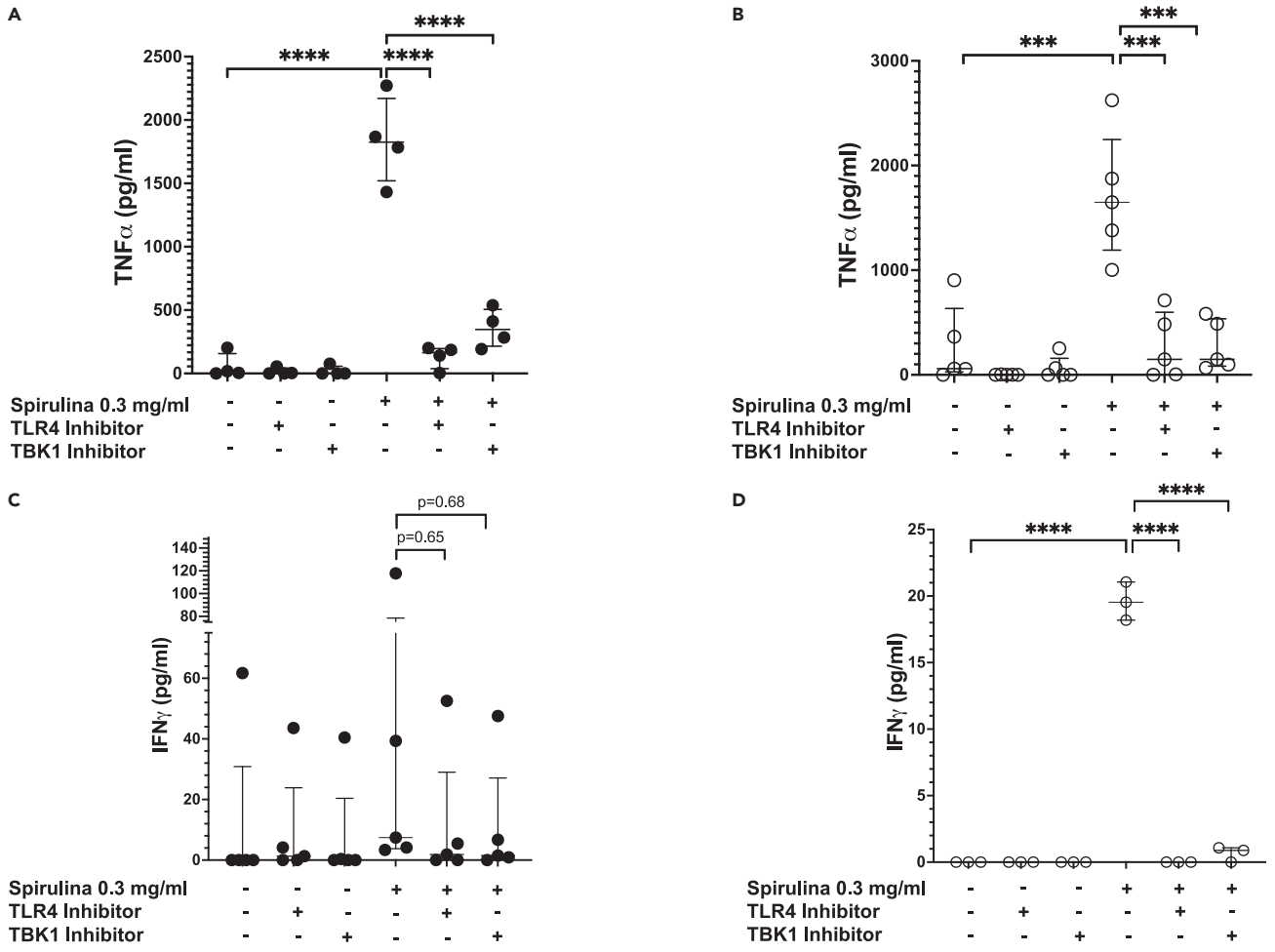


Figure 2. TNFα and IFNγ secretion by Spirulina-stimulated PBMCs in patients with DM and HCs in the presence of TBK1 and TLR4 inhibitor by ELISA

(A) TNFα supernatant levels in DM Spirulina-stimulated PBMCs pre-stimulated with TBK1 or TLR4 inhibition.

(B) TNFα supernatant levels in Healthy Control PBMCs with TBK1 or TLR4 inhibition.

(C) IFNγ supernatant levels in DM Spirulina-stimulated PBMCs pre-stimulated with TBK1 or TLR4 inhibition.

(D) IFNγ supernatant levels in Healthy Control PBMCs with TBK1 or TLR4 inhibition. Bars show *p < 0.05, **p < 0.01, ****p < 0.0001. Lines represent the median and interquartile range.

When evaluating IFNγ production in DM PBMCs, CMs and moDCs had the greatest average percentage increase (86.7 ± 6.4% and 90.5 ± 4.9%, respectively) from baseline compared to all cell lineages (Figure 3G), with the percent IFNγ$^+$ CMs increasing from 8.3 ± 3.5% to 94.8 ± 4.2% ($p < 0.0001$) and moDCs increasing from 0.8 ± 0.6% to 91.3 ± 5.2% ($p < 0.0001$) with 0.3 mg/mL Spirulina (Figures 3H and 3I). While Spirulina 0.3 mg/mL also increased the percentage of IFNγ$^+$ CMs (4.1 ± 1.5% to 14.4 ± 6.2%) and moDCs (1.0 ± 0.3% to 21.7 ± 5.5%) in HCs, the increase was still significantly lower compared to Spirulina-treated DM populations ($p < 0.0001$ for both CMs and moDCs, respectively) (Figures 3H and 3I). There were no differences in the percent of IFNγ$^+$ CMs or moDCs at baseline between DM and HC ($p > 0.99$).

Notably, among patients with DM, Spirulina 0.3 mg/mL also increased the percentage of IFNγ$^+$ CD4, CD11c$^+$ cDCs, CD123 pDCs, IMs, and nCMs compared to baseline ($p < 0.05$). Among HCs, Spirulina also dose-dependently increased the percentage of IFNγ$^+$ CD11c$^+$ cDCs ($p < 0.01$) and CD123$^+$ pDCs ($p < 0.01$) at 1 mg/mL (Figures S3A–S3F).

Inhibition of TANK-binding kinase 1 or Toll-like receptor 4 activation does not affect interferon β activity, but it decreases Spirulina-induced production of tumor necrosis factor α in classical monocytes and monocyte-derived dendritic cells in dermatomyositis

Using different inhibitors of the inflammatory cascade, including STING, TLR4, and TBK1, we were able to further characterize the mechanisms by which Spirulina activates inflammatory cytokine production at the cellular level.

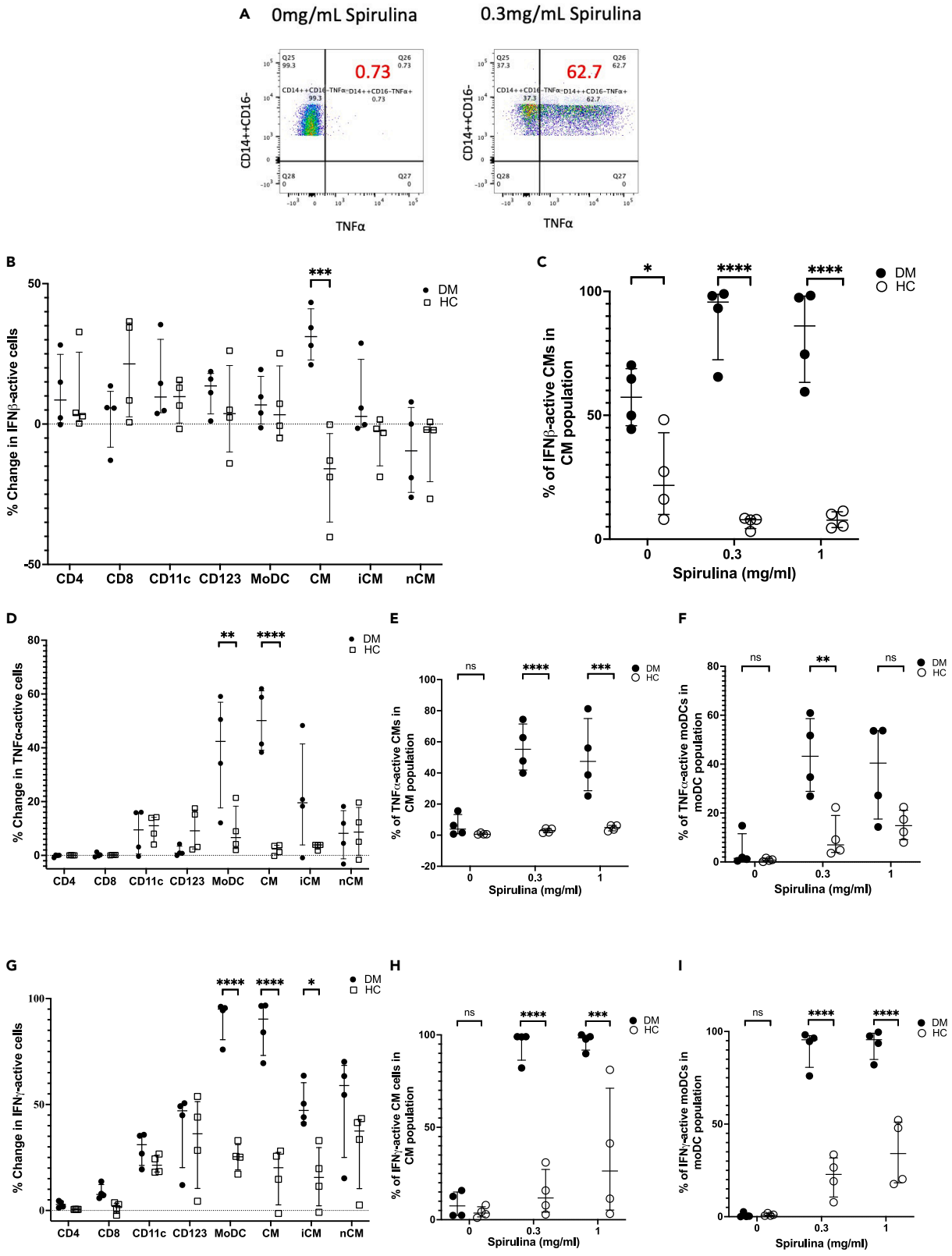

A
0mg/mL Spirulina
0.3mg/mL Spirulina
0.73
62.7
CD14++CD16-
TNFα
B
% Change in IFNβ-active cells
CD4
CD8
CD11c
CD123
MoDC
CM
iCM
nCM
DM
HC
C
% of IFNβ-active CMs in CM population
Spirulina (mg/ml)
D
% Change in TNFα-active cells
E
% of TNFα-active CMs in CM population
F
% of TNFα-active moDCs in moDC population
G
% Change in IFNγ-active cells
H
% of IFNγ-active CM cells in CM population
I
% of IFNγ-active moDCs in moDC population

Figure 3. Eight cell lineages (CD4, CD8, CD11c, CD123, CD14++CD16$^-$ CMs, CD11c+CD14$^+$ moDCs, CD14++CD16$^+$ IMs, and CD14$^+$CD16++ nCMs in Spirulina-stimulated DM and HC PBMCs evaluated using flow cytometry

(A) Representative gating strategy used when analyzing the percentage-of-parent at zero stimulation and with 0.3 mg/mL of Spirulina.

(B) Percent change in IFNβ-active cells from baseline to 0.3 mg/mL Spirulina stimulation between HC and DM of each cell type.

(C) Percent of CMs secreting IFNβ was measured in DMs and HCs at baseline, 0.3 mg/mL, and 1 mg/mL Spirulina stimulation.

(D) Percent change in TNFα-active cells from baseline to 0.3 mg/mL Spirulina stimulation as well as between HC and DM.

(E and F) Percent of CMs and moDCs secreting TNFα at baseline, 0.3 mg/mL, and 1 mg/mL Spirulina stimulation.

(G) The percent change of IFNγ-secreting cells from baseline to 0.3 mg/mL Spirulina stimulation.

(H and I) Percent of CMs and moDCs secreting IFNγ was measured in DMs and HCs at baseline, 0.3 mg/mL, and 1 mg/mL Spirulina stimulation. *$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$. Lines represent the median and interquartile range.

When evaluating the Spirulina-stimulated IFNβ-active CM response, STING, TBK1 or TLR4 inhibition did not influence activated CMs ($p > 0.99$ for all inhibitors) (Figure 4A). In contrast, for TNFα$^+$ CMs, TLR4 inhibition significantly decreased Spirulina-stimulated CMs in patients with DM, with the percent of CMs decreasing from a mean 56.2 ± 7.7% at 0.3 mg/mL Spirulina stimulation to 20.4 ± 4.4% of CMs with TLR4 inhibition ($p < 0.05$). STING inhibition did not inhibit the effect ($p > 0.99$), and TBK1 inhibition trended downwards (mean 31.9 ± 8.6% of CMs, $p = 0.27$) (Figure 4B). The mean percent of moDCs secreting TNFα also significantly decreased after either TBK1 or TLR4 inhibition (Figure 4C). TNFα$^+$ moDCs decreased from 43.6 ± 7.8% of the total moDC population to 18.7 ± 3.7% ($p < 0.05$) with TBK1 inhibition and to 10.0 ± 4.7% ($p < 0.01$) with TLR4 inhibition. STING inhibition had no effect ($p > 0.99$).

Inhibition of TANK-binding kinase 1 or Toll-like receptor 4 activation decreases Spirulina-induced production of interferon γ in classical monocytes and monocyte-derived dendritic cells in dermatomyositis

For IFNγ$^+$ cells, pre-treatment with TLR4 inhibitor decreased the percentage of Spirulina-stimulated CMs secreting IFNγ from 95.0 ± 4.3% of CMs to 76.9 ± 9.8% of CMs, although this effect was not significant ($p = 0.23$) (Figure 4D). However, median fluorescent intensity (MFI) did significantly decrease. MFI of IFNγ$^+$ CMs increased 39-fold after 0.3 mg/mL Spirulina stimulation ($p < 0.0001$), with TLR4 inhibition reducing Spirulina's effect by two-thirds ($p < 0.0001$) (Figure 4E). Spirulina-stimulated CM MFI trended downwards with TBK1 inhibition (28,153.0 ± 3706.6 a.u at 0.3 mg/mL Spirulina vs. 18,736.0 ± 3368.1 a.u, $p = 0.16$), and STING inhibition had no effect ($p > 0.99$). TLR4 inhibition significantly decreased the percentage of Spirulina-induced moDCs producing IFNγ. The mean percentage of moDCs secreting IFNγ decreased from 91.30 ± 5.2% with 0.3 mg/mL Spirulina, to 58.0 ± 12.4% with TLR4 inhibitor ($p < 0.01$). Pre-treatment with STING inhibitor or TBK1 inhibitor did not attenuate the response of Spirulina-treated cells ($p > 0.99$ for both inhibitors) (Figure 4F). MFI of IFNγ$^+$ moDCs also significantly increased with 0.3 mg/mL Spirulina ($p < 0.0001$) and significantly decreased with TLR4 inhibition ($p < 0.01$), but not with with STING nor TBK1 inhibition (Figure 4G).

Inhibition of stimulator of interferon genes, TANK-binding kinase 1, or Toll-like receptor 4 has minimal effect on inflammatory cytokine activity of classical monocytes and monocyte-derived dendritic cells in healthy controls peripheral blood mononuclear cells

When evaluating the response of inhibitors in the HC population, STING, TBK1, and TLR4 inhibition did not affect the IFNβ response (Figure S4A). In contrast to the DM population, STING, TBK1, and TLR4 inhibition did not affect the percent of Spirulina-stimulated TNFα$^+$ CMs (Figure S4B) in HCs. TLR4 inhibition trended toward decreasing the percent of Spirulina-stimulated TNFα$^+$ moDCs, with 10.0 ± 4.3% of moDCs producing TNFα at 0.3 mg/mL Spirulina compared to 2.0 ± 0.6% with TLR4 inhibitor present, although the effects were not significant ($p = 0.31$) (Figure S4C). STING, TBK1, and TLR4 inhibition did not decrease the percentage of IFNγ$^+$ CMs ($p > 0.99$, $p > 0.95$, $p = 0.88$, respectively) or percent of IFNγ$^+$ moDCs (>0.95 for all inhibitors) (Figures S4D and S4E). These results are not surprising, given that Spirulina did not significantly increase cytokine activity in HCs; therefore, abrogation via TBK1 and TLR4 inhibition is unlikely.

Similar immune cells are present in the skin of patients with dermatomyositis with and without Spirulina exposure

We sought to characterize the immune compartment of DM skin from patients with Spirulina-associated DM and patients with no history of Spirulina consumption using IMC. Unsupervised clustering yielded 15 distinct cell populations in DM and Spirulina-associated DM samples, including CD4 T, CD8 T, naive CD8 T, γδ T, Treg, CD20 B, conventional dendritic cells (cDC), cDC type 1 (cDC1) and 2 (cDC2), CD14$^+$CD16$^+$ Macrophages (Mac), CD14$^+$CD16++ Mac, CD14$^+$CD16$^-$ Mac, CD68$^+$ Mac, CD163+ Mac, plasmacytoid dendritic cells (pDC), neutrophils, CD56$^+$, and endothelial cells.

To identify potential cell types recruited to Spirulina-associated DM skin, we compared both cell counts (Figure 5A) and percentage of total leukocytes between DM and Spirulina-associated DM. No significant differences were identified between the cell infiltrates ($p > 0.05$). We then sought to identify potentially upregulated cytokines or inflammatory pathways in Spirulina-associated DM by using mean intracellular staining (Figure S5A). No significant differences in expression were found ($p > 0.05$). At the cell-type level, heatmaps were generated to visually compare the relative intracellular MPI for each cell cluster between DM and Spirulina-associated DM (Figure S6). Notably, the staining patterns between DM and Spirulina-associated DM were similar despite substantial patient heterogeneity within each group.

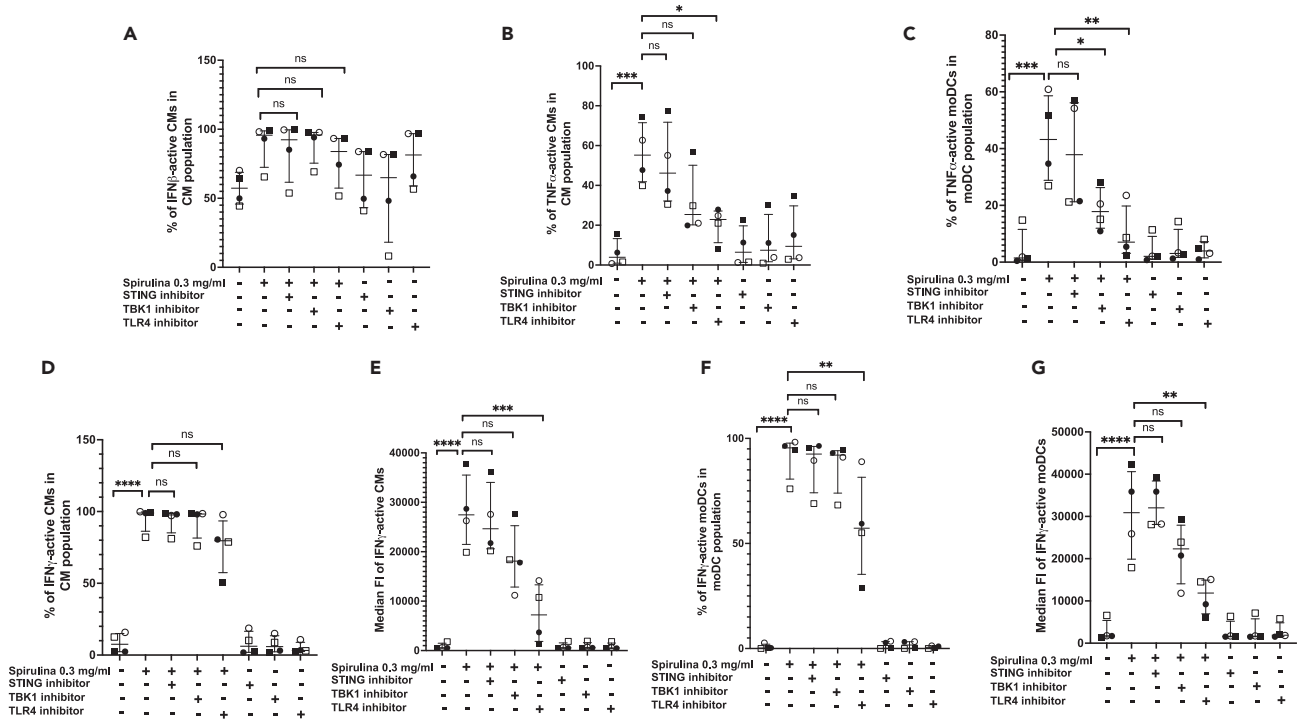


Figure 4. TNFα and IFNγ secretion by Spirulina-stimulated PBMCs in patients with DM and HCs in the presence of STING, TBK1 and TLR4 inhibitor by flow cytometry

(A) Percent of CMs secreting IFNβ was measured in DM Spirulina-stimulated PBMCs pre-treated with inhibitors to STING, TBK1 and TLR4.

(B) Percent of CMs secreting TNFα was measured in DM Spirulina-stimulated PBMCs pre-treating with STING, TBK1 and TLR4 inhibitors.

(C) Percent of moDCs secreting TNFα was measured in DM Spirulina-stimulated PBMCs pre-treated with STING, TBK1 and TLR4 inhibitors.

(D and E) Percent of CMs and MFI of CMs secreting IFNγ was measured in DM Spirulina-stimulated PBMCs pre-treated with STING, TBK1 and TLR4 inhibitors.

(F and G) Percent of moDCs and MFI of moDCs secreting IFNγ was measured in DM Spirulina-stimulated PBMCs pre-treated with STING, TBK1 and TLR4 inhibitors. *$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$. Lines represent the median and interquartile range.

Since IFNγ priming has been shown to enhance phagocyte expression of TLR4 (30), we sought to determine whether IFNγ and TLR4 correlated in DM and Spirulina-associated DM skin. We found TLR4 expression correlated more with IFNγ levels in Spirulina-associated DM than in non-Spirulina associated DM skin ($r = 0.97$ vs. 0.73 (Figures S5B and S5C). The correlation in DM skin was entirely driven by one patient. We then evaluated cellular expression of TLR4 in DM and Spirulina-associated DM and found that Spirulina-associated DM skin cDC2s expressed more TLR4 ($p < 0.05$) than DM (Figure S5D). Moreover, our data demonstrated that cDC2 expression of TLR4 correlated with total IFNγ in Spirulina-associated DM ($r = 1.0$) but not in DM ($p > 0.05$) (Figures S5E and S5F).

DISCUSSION

This study highlights the immunostimulatory effects that Spirulina, a popular herbal supplement, has on PBMCs in patients with DM *in vitro*. We identify possible mechanisms of action to elucidate how and why the immune response may differ between patients with DM and HC. We found that water-soluble Spirulina significantly stimulates inflammatory cytokine production, including IFNβ, TNFα, and IFNγ by DM PBMCs through TLR4 and TBK1 activation, and that this response in the blood is primarily driven by the activation of CMs and moDCs. Spirulina induced a significantly greater response in CM and moDC activation in patients with DM than in HCs, with the percentage of CMs and moDCs secreting IFNγ as well as the median fluorescent intensity (MFI) of the signal increasing dramatically. The differential responses in these two cell types suggests that DM cells may be pre-primed, providing a possible explanation for why only some patients flare or develop DM.

As a cyanobacteria, Spirulina contains LPS similar to that of gram-negative bacteria with significant toxicity, and LPS makes up approximately 1.6% of dry Spirulina.[30] TLR4 is the signaling receptor for LPS.[31] When LPS binds to TLR4, two different pathways can be activated, the myeloid differentiation primary response 88 (Myd88)-dependent pathway, leading to NF-κB activation and TNFα production, or the MyD88-independent pathway, involving the adaptor proteins TIR-domain-containing adaptor-inducing interferon-β (TRIF), TRIF- related adaptor molecule (TRAM), and TBK1, and initiating a type-1 IFN response and late NF-κB activation.[32,33]

TLR4 activation by Spirulina may be partially mediated by LPS in addition to other immunostimulatory components. Prior work found that Spirulina activates the TLR4 MyD88-dependent pathway,[34] while an isolated immunostimulatory polysaccharide in Spirulina, Immulina, was

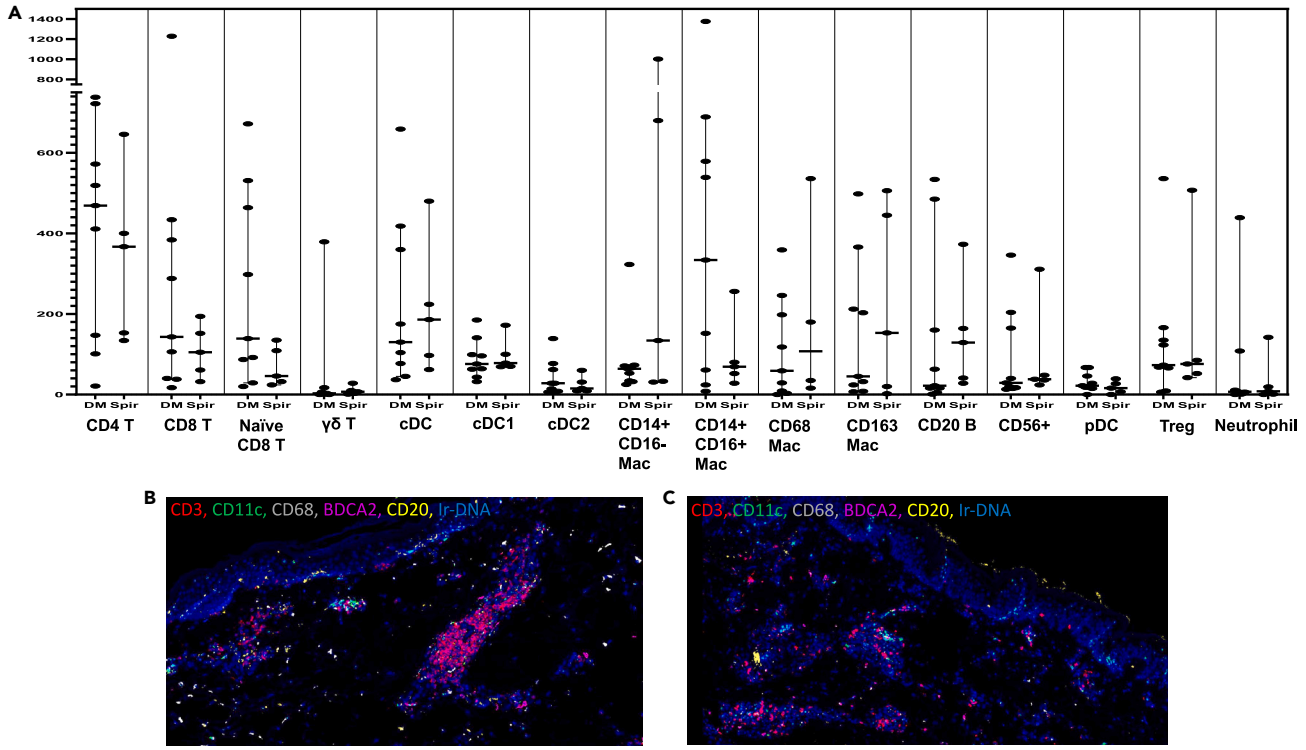


Figure 5. Imaging Mass Cytometry of patients with DM with and without Spirulina use

(A) Cell counts in DM and Spirulina-associated DM skin biopsies. The center line represents the median, the box contains the 25th to 75th percentiles, and the whiskers contain the minimum and maximum values.

(B and C) Representative lesional biopsies of DM (b) and Spirulina-associated DM (c) showing compositions of CD3 (red), CD11c (green), CD68 (gray), BDCA2 (magenta), and CD20 (yellow). Counterstain is with Iridium-191 DNA intercalator (blue).

found to primarily activate monocytes via Toll-like receptor 2 (TLR2).[16] As TLR4 inhibitor significantly attenuated Spirulina-induced TNFα and IFNγ production at the cellular level, our results support the finding that Spirulina activates TLR4, likely via the MYD88-dependent pathway to activate NF-κB as well as the LPS-induced MyD88-independent pathway, leading to interferon production.[35] While some of Spirulina's immunostimulatory effects may be due to LPS, this does not completely explain our results. We have demonstrated previously with IsaLean (another herbal supplement) and with our present data, that TLR4 inhibition nearly completely abrogates TNFα production *in vitro*.[18] This contrasts with LPS, which signals through both TLR4 and TLR2, suggesting these are two similar compounds with somewhat different signaling pathways.

Moreover, the significant decreases in IFNγ production by moDCs and MFI by both CMs and moDCs with TLR4 inhibition, but not TBK1 inhibition, suggest that Spirulina-induced IFNγ production may be primarily via the MyD88-dependent pathway, where TBK1 is not involved. In contrast, Spirulina-induced TNFα production may be at least partially mediated via the MyD88-independent pathway and late NF-κB response as well, given that TBK1 inhibition decreased the percentage of Spirulina-stimulated TNFα^{+} moDCs.

While the role of CMs and moDCs in DM is still only poorly understood, CMs play an integral role in the innate inflammatory response, including phagocytosis, antigen presentation, and cytokine production,[36] and they can differentiate into monocyte-derived macrophages and moDCs.[37–39] We previously reported that lesional DM skin has increased numbers of CD14^{+} and CD14^{+}CD16^{+} cells compared to HC skin ($p < 0.05$). In addition, the CD14^{+} population positively correlated with disease severity, measured with the Cutaneous Dermatomyositis Disease Area and Severity Index (CDASI) ($p < 0.05$),[22] implicating CMs and moDCs in DM disease pathogenesis.

Our results using IMC show that although Spirulina preferentially activates certain cells, the inflammatory infiltrate of Spirulina-associated DM is very similar to that of DM from other causes. The IMC results reveal a great degree of heterogeneity in the skin of patients with DM, as we have previously shown *in vitro*, yet no significant differences in our relatively small cohort.[16]

Here we demonstrate that IFNγ is positively correlated with TLR4 to a greater extent in lesional Spirulina-associated DM skin than in idiopathic DM alone. Spirulina's TLR4-activating properties and evidence of synergy between TLR4 and IFNγ activation in the inflammatory response may contribute to the differential cellular activation seen in patients with DM compared to HCs. Aberrant activation of toll-like receptors by endogenous or exogenous ligands is thought to be integral to the initiation and subsequent propagation of an autoimmune response.[40] Bosisio et al. found that IFNγ increased mRNA and surface expression of TLR4. IFNγ-primed monocytes subsequently had a greater response to LPS stimulation, with increased NF-κB binding activity, kinase activity downstream of MyD88 adaptor protein, and

TNFα production,[41] suggesting that TLR4 upregulation may account for previously described IFNγ macrophage priming effects.[42–44] Moreover, concomitant IFNγ and LPS stimulation of TLR4 leads to the differential regulation of enhancers and super-induction of inflammatory genes, thereby significantly increasing the inflammatory response.[44]

Increased TLR4 activation may contribute to DM pathogenesis. In a study of monocyte populations in the sera of HCs, DMs, and patients with polymyositis (PMs), classical, intermediate, and non-classical monocytes from DM sera and PM sera expressed greater levels of TLR4 compared to HCs, irrespective of disease activity or treatment course.[45] Similarly, in DM and PM muscle infiltrates, TLR2, TLR4, and TLR9 expression levels were significantly greater compared to HCs, with TLR4 and TLR9 expression levels in muscle positively correlating with IFNγ, IL-4, Interleukin 17 (IL-17), and TNFα expression.[26] Moreover, in DM skin infiltrate, inflammatory pathway markers and cytokines within the TLR4 pathway, including Interferon regulatory transcription factor (IRF)-3, TBK1, IFNβ, and IFNγ, were all upregulated compared to HCs.[22]

Our studies utilizing DM PBMC's *in vitro* and examining inflammatory cells/pathways *in vivo* through IMC studies suggest that Spirulina may promote autoimmunity via the production of IFNγ in CMs and moDCs. Potential autocrine induction of TLR4 expression in both the blood and skin implicates Spirulina in the development of DM in some susceptible patients. Our findings, taken together with the current literature, suggest that Spirulina may trigger disease onset or flare in susceptible patients. To our knowledge, this is the first time that the mechanism by which Spirulina increases cytokine levels has been studied in autoimmune skin disease patients, in general, and in patients with DM specifically, thus identifying a possible pathomechanism between Spirulina consumption and DM onset/flare. Further characterization of Spirulina's immunostimulatory role is critical to better understand how Spirulina upregulates inflammatory cytokines in patients with DM, and to ultimately inform physicians and patients to make health-conscious decisions and guide the clinical management of patients with autoimmune skin diseases.

Limitations of the study

Our study has several limitations. Firstly, three of the four patients with DM were on potent immunomodulating medications, including prednisone, tofacitinib, hydroxychloroquine, and mycophenolate mofetil (Table 1), and therefore the measured immune response in these *in vitro* studies may be diminished. However, substantial differences in activation and inflammatory cytokine production were still detected between patients with DM and HCs despite substantial immunosuppression. Second, patients were only tested after DM onset and diagnosis, so although we observed how the HC and DM response to Spirulina is different, we are unable to assess what was unique about the individual person pre-Spirulina induction. Third, relatively high concentrations of Spirulina were utilized. Prior studies have used the isolated functional compound, Immulina, which has immunostimulatory properties[17–19] and makes up just 0.5% of the dry weight of Spirulina.[18] We evaluated the overall effect of all water-soluble components of the herbal supplement Spirulina, rather than just the effect of one small, crude Spirulina extract. Spirulina 0.3 mg/mL is also similar to physiologic Spirulina levels post-consumption. Our exploratory analysis comparing DM and Spirulina-associated DM using IMC utilized a small sample size because of the few Spirulina-associated DM subjects with available treatment-naïve biopsies. More robust studies are needed to verify these results.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- RESOURCE AVAILABILITY
  - Lead contact
  - Materials availability
  - Data and code availability
- EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS
  - Patients
- METHOD DETAILS
  - Spirulina preparation
  - Determination of Spirulina concentration used in experiments
  - Peripheral blood mononuclear cell (PBMC) isolation, stimulation with Spirulina, and culture
  - Enzyme-linked immunosorbent assay (ELISA)
  - Flow cytometry
  - Imaging mass cytometry
- QUANTIFICATION AND STATISTICAL ANALYSIS

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2023.108355.

Table 1. Demographics, medications and DM classification table

Experiment	Sex	Age	Race/Ancestry	Current medications	DM antibody type	CDASI	Predominant disease type
FACS, ELISA	F	47	White, non-Hispanic	Prednisone 1 mg/day, Hydroxychloroquine 300 mg/day	Not available	5	Skin and Muscle[a]
FACS	F	43	White, non-Hispanic	Mycophenolate Mofetil, Hydroxychloroquine 400 mg/day	Not available	9	Skin and Muscle
FACS, ELISA	F	53	White, non-Hispanic	Mycophenolate Mofetil, Tofacitinib[b]	MDA5+	12	Skin
FACS[c]	M	30	White, non-Hispanic	None	Not available	14	Skin
IMC DM	F	59	White, non-Hispanic	Prednisone 80mg/day	Not available	Not available	Skin, muscle, and lung
IMC DM	F	39	White, non-Hispanic	Prednisone unspecified dose	Not available	Not available	Skin and muscle
IMC DM	F	70	White, non-Hispanic	None	Not available	26	Skin
IMC DM	F	56	White, non-Hispanic	Hydroxychloroquine 400mg/day, Mycophenolate Mofetil 2000mg/day	Not available	26	Skin
IMC DM	F	50	White, non-Hispanic	None	Not available	Not available	Skin
IMC DM	F	66	white, non-Hispanic	None	Not available	18	Skin
IMC DM	F	44	Black, non-Hispanic	Prednisone 40mg/day, Mycophenolate Mofetil 3000mg/day	PL-7[d]	3	Skin
IMC DM	F	54	White, non-Hispanic	None	Not available	Not available	Skin
IMC Spirulina-associated DM	F	48	White, non-Hispanic	None	Not available	Not available	Skin and muscle
IMC Spirulina-associated DM	F	51	White, non-Hispanic	Hydroxychloroquine 400mg/day	Not available	9	Skin
IMC Spirulina-associated DM	F	65	White, non-Hispanic	None	Not available	Not available	Skin
IMC Spirulina-associated DM	F	34	White, non-Hispanic	Hydroxychloroquine 400mg/day	ANA, RNP	10	Skin
IMC Spirulina-associated DM	F	78	Asian, non-Hispanic	None	Not available	Not available	Skin
ELISA DM	M	76	White, non-Hispanic	None	Not available	6	skin and muscle
ELISA DM	F	77	Black, non-Hispanic	None	Not available	0	skin and muscle

(*Continued on next page*)

Table 1. *Continued*

Experiment	Sex	Age	Race/Ancestry	Current medications	DM antibody type	CDASI	Predominant disease type
ELISA DM	F	53	White, non-Hispanic	Hydroxychloroquine 400 mg/day	Not available	6	skin
ELISA DM	F	67	White, non-Hispanic	Mycophenolate Mofetil 3000 mg/day, Hydroxychloroquine 300 mg/day	+SAE1	17	skin and muscle
ELISA DM	F	55	White, non-Hispanic	Hydroxychloroquine 300 mg/day, Mycophenolate Mofetil 2500 mg/day	Not available	5	skin
ELISA DM	F	47	White, non-Hispanic	Hydroxychloroquine 400 mg/day	Not available	15	skin and muscle
ELISA DM	F	62	White, non-Hispanic	None	Not available	10	skin
ELISA DM	F	53	White, Hispanic	25 mg Methotrexate sc/week	Not available	Not available	Skin, muscle, and lung
ELISA DM	M	73	White, non-Hispanic	Hydroxychloroquine 400 mg/day	Not available	10	Skin and Muscle
ELISA DM	F	75	White, non-Hispanic	Hydroxychloroquine 200 mg/day, Chloroquine 250 mg/day, Quinacrine 100mg/day, Mycophenolate Mofetil 1000mg/day	PL 7 Ab	12	Skin, muscle, and lung
ELISA DM	F	61	White, non-Hispanic	Hydroxychloroquine 300mg/day	Jo-1+	6	Skin, muscle, and lung
ELISA DM	M	49	White, non-Hispanic	Dapsone 100 mg/day	Not available	15	Skin and Muscle
ELISA DM	F	75	White, non-Hispanic	Mycophenolate Mofetil 3000mg/day	TIF-1-gamma	9	Skin and lung
ELISA DM	F	84	White, non-Hispanic	Mycophenolate Mofetil 500 mg BID	TIF-1-gamma, PL-7	15	Skin
ELISA DM	F	65	White, non-Hispanic	None	RO-52	35	Skin
ELISA DM[c]	M	32	White, non-Hispanic	Prednisone, 60, 40, 20 over 15 days.	Not available	24	Skin
ELISA DM	F	29	White, non-Hispanic	Prednisone 5 mg/day and Azathioprine 100 mg/day	Not available	9	Skin and muscle
ELISA DM	F	65	White, non-Hispanic	Prednisone over 15 days.	+anti-P155/140 antibody	8	skin
ELISA DM	F	59	White, non-Hispanic	Mycophenolate Mofetil 2000 mg/day	Not available		Skin and Muscle
ELISA DM	F	46	Black, non-Hispanic	Intravenous Immunoglobulin	PL 7 Ab	4	Skin
ELISA DM	F	57	White, non-Hispanic	Hydroxychloroquine 400 mg/day	Not available	Not available	Skin and Muscle

FACS, fluorescence-activated cell sorting/flow cytometry; IMC, Imaging Mass Cytometry; ELISA, Enzyme linked immunosorbent assay.
[a]Subjective muscle involvement although muscle enzyme serologies within normal limits.
[b]Patient had not taken Tofacitinib or Mycophenolate Mofetil for 2 weeks prior to blood draw.
[c]Patient data collected from two separate visits for two separate experiments.
[d]PL-7 was transiently positive.

ACKNOWLEDGMENTS

This work was supported by the NIH TL1 grant number TR001880 (CEB) as well as the NIH (NIAMS) R01 AR076766. This work was also supported by the United States Department of Veterans Affairs (Veterans Health Administration, Office of Research and Development and Biomedical Laboratory Research and Development) (VPW). This research was supported by Core A of the Penn Skin Biology and Diseases Resource-based Center, funded by 1P30AR069589 - 01 (Millar).

AUTHOR CONTRIBUTIONS

Study conceptualization: VPW, CEB, YL, DD, TV, and JP; sata curation and analysis: VPW, DD, CEB, YL, AR, SM, MG, TV, and JP; article writing: CEB, DD, TV, JP, and VPW; article review and editing: DD, CEB, VPW, YL, AR, SM, TV, EK, and MG.

DECLARATION OF INTERESTS

The authors declare no conflicts of interest.

INCLUSION AND DIVERSITY

We support inclusive, diverse, and equitable conduct of research.

Received: October 3, 2022
Revised: September 8, 2023
Accepted: October 24, 2023
Published: October 28, 2023

REFERENCES

1. Deng, R., and Chow, T.-J. (2010). Hypolipidemic, antioxidant and antiinflammatory activities of microalgae Spirulina. Cardiovasc. Ther. *28*, e33–e45. https://doi.org/10.1111/j.1755-5922.2010.00200.x.
2. Karkos, P.D., Leong, S.C., Karkos, C.D., Sivaji, N., and Assimakopoulos, D.A. (2011). Spirulina in clinical practice: evidence-based human applications. Evid. Based. Complement. Alternat. Med. *2011*, 531053–531054. https://doi.org/10.1093/ecam/nen058.
3. Kessler, R.C., Davis, R.B., Foster, D.F., Van Rompay, M.I., Walters, E.E., Wilkey, S.A., Kaptchuk, T.J., and Eisenberg, D.M. (2001). Long-term trends in the use of complementary and alternative medical therapies in the United States. Ann. Intern. Med. *135*, 262–268.
4. Ravishankar, A., Yan, D., Bax, C., Concha, J.S., Shields, B., Pappas-Taffer, L., Feng, R., Okawa, J., and Werth, V. (2020). Immunostimulatory Herbal Supplement Use Is More Common Among Patients with Dermatomyositis (WILEY). 111 RIVER ST, HOBOKEN 07030-5774, NJ USA.
5. Konno, T., Umeda, Y., Umeda, M., Kawachi, I., Oyake, M., and Fujita, N. (2011). [A case of inflammatory myopathy with widely skin rash following use of supplements containing Spirulina]. Rinsho Shinkeigaku *51*, 330–333.
6. Lee, A.N., and Werth, V.P. (2004). Activation of autoimmunity following use of immunostimulatory herbal supplements. Arch. Dermatol. *140*, 723–727. https://doi.org/10.1001/archderm.140.6.723.
7. Kraigher, O., Wohl, Y., Gat, A., and Brenner, S. (2008). A mixed immunoblistering disorder exhibiting features of bullous pemphigoid and pemphigus foliaceus associated with Spirulina algae intake. Int. J. Dermatol. *47*, 61–63. https://doi.org/10.1111/j.1365-4632.2007.03388.x.
8. Ravishankar, A., Bax, C., Grinnell, M., Yan, D., Feng, R., Okawa, J., and Werth, V. (2021). 429 Spirulina use and its temporal association with dermatomyositis exacerbation. J. Invest. Dermatol. *141*, S74.
9. Ravishankar, A., Bax, C.E., Grinnell, M., Yan, D., Concha, J.S., Pappas-Taffer, L., Shields, B.E., Dany, M., Clark, A.K., Feng, R., et al. (2022). Frequency of immunostimulatory herbal supplement use among patients with autoimmune skin disease. J. Am. Acad. Dermatol. *87*, 1093–1095. https://doi.org/10.1016/j.jaad.2021.12.050.
10. Finamore, A., Palmery, M., Bensehaila, S., and Peluso, I. (2017). Antioxidant, immunomodulating, and microbial-modulating activities of the sustainable and ecofriendly Spirulina. Oxid. Med. Cell. Longev. *2017*, 3247528. https://doi.org/10.1155/2017/3247528.
11. Ngo-Matip, M.E., Pieme, C.A., Azabji-Kenfack, M., Moukette, B.M., Korosky, E., Stefanini, P., Ngogang, J.Y., and Mbofung, C.M. (2015). Impact of daily supplementation of Spirulina platensis on the immune system of naïve HIV-1 patients in Cameroon: a 12-months single blind, randomized, multicenter trial. Nutr. J. *14*, 70. https://doi.org/10.1186/s12937-015-0058-4.
12. Azabji-Kenfack, M., Dikosso, S.E., Loni, E.G., Onana, E.A., Sobngwi, E., Gbaguidi, E., Kana, A.L.N., Nguefack-Tsague, G., Von der Weid, D., Njoya, O., and Ngogang, J. (2011). Potential of Spirulina platensis as a nutritional supplement in malnourished HIV-infected adults in sub-saharan Africa: a randomised, single-blind study. Nutr. Metab. Insights *4*, 29–37. https://doi.org/10.4137/nmi.S5862.
13. Ge, Y., Kang, Y.K., Dong, L., Liu, L.H., and An, G.Y. (2019). The efficacy of dietary Spirulina as an adjunct to chemotherapy to improve immune function and reduce myelosuppression in patients with malignant tumors. Transl. Cancer Res. *8*, 1065–1073. https://doi.org/10.21037/tcr.2019.06.13.
14. Hirahashi, T., Matsumoto, M., Hazeki, K., Saeki, Y., Ui, M., and Seya, T. (2002). 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. *2*, 423–434. https://doi.org/10.1016/S1567-5769(01)00166-7.
15. Mishima, T., Murata, J., Toyoshima, M., Fujii, H., Nakajima, M., Hayashi, T., Kato, T., and Saiki, I. (1998). Inhibition of tumor invasion and metastasis by calciumspirulan (Ca-SP), a novel sulfated polysaccharide derived from a blue-green alga, Spirulina platensis. Clin. Exp. Metastasis *16*, 541–550.
16. Balachandran, P., Pugh, N.D., Ma, G., and Pasco, D.S. (2006). Toll-like receptor 2-dependent activation of monocytes by Spirulina polysaccharide and its immune enhancing action in mice. Int. Immunopharmacol. *6*, 1808–1814. https://doi.org/10.1016/j.intimp.2006.08.001.
17. Nielsen, C.H., Balachandran, P., Christensen, O., Pugh, N.D., Tamta, H., Sufka, K.J., Wu, X., Walsted, A., Schjørring-Thyssen, M., Enevold, C., and Pasco, D.S. (2010). Enhancement of natural killer cell activity in healthy subjects by Immulina®, a Spirulina extract enriched for braun-type lipoproteins. Planta Med. *76*, 1802–1808. https://doi.org/10.1055/s-0030-1250043.
18. Pugh, N., Ross, S.A., ElSohly, H.N., ElSohly, M.A., and Pasco, D.S. (2001). Isolation of three high molecular weight polysaccharide preparations with potent immunostimulatory activity from Spirulina platensis, aphanizomenon flos-aquae and Chlorella pyrenoidosa. Planta Med. *67*, 737–742. https://doi.org/10.1055/s-2001-18358.
19. Grzanna, R., Polotsky, A., Phan, P.V., Pugh, N., Pasco, D., and Frondoza, C.G. (2006). Immolina, a high–molecular-weight polysaccharide fraction of Spirulina, enhances chemokine expression in human monocytic THP-1 cells. J. Altern. Complement. Med. *12*, 429–435. https://doi.org/10.1089/acm.2006.12.429.

20. Mao, T.K., Van De Water, J., and Gershwin, M.E. (2000). Effect of spirulina on the secretion of cytokines from peripheral blood mononuclear cells. J. Med. Food *3*, 135–140. https://doi.org/10.1089/jmf.2000.3.135.
21. Løbner, M., Walsted, A., Larsen, R., Bendtzen, K., and Nielsen, C.H. (2008). Enhancement of human adaptive immune responses by administration of a high-molecular-weight polysaccharide extract from the cyanobacterium Arthrospira platensis. J. Med. Food *11*, 313–322. https://doi.org/10.1089/jmf.2007.564.
22. Patel, J., Maddukuri, S., Li, Y., Bax, C., and Werth, V.P. (2021). Highly multiplexed mass cytometry identifies the immunophenotype in the skin of dermatomyositis. J. Invest. Dermatol. *141*, 2151–2160. https://doi.org/10.1016/j.jid.2021.02.748.
23. Kao, L., Chung, L., and Fiorentino, D.F. (2011). Pathogenesis of dermatomyositis: role of cytokines and interferon. Curr. Rheumatol. Rep. *13*, 225–232. https://doi.org/10.1007/s11926-011-0166-x.
24. Zeidi, M., Chansky, P.B., and Werth, V.P. (2019). Acute onset/flares of dermatomyositis following ingestion of IsaLean herbal supplement: clinical and immunostimulatory findings. J. Am. Acad. Dermatol. *80*, 801–804. https://doi.org/10.1016/j.jaad.2018.08.019.
25. Pugh, N.D., Edwall, D., Lindmark, L., Kousoulas, K.G., Iyer, A.V., Haron, M.H., and Pasco, D.S. (2015). Oral administration of a Spirulina extract enriched for Braun-type lipoproteins protects mice against influenza A (H1N1) virus infection. Phytomedicine *22*, 271–276. https://doi.org/10.1016/j.phymed.2014.12.006.
26. Kim, G.T., Cho, M.L., Park, Y.E., Yoo, W.H., Kim, J.H., Oh, H.J., Kim, D.S., Baek, S.H., Lee, S.H., Lee, J.H., et al. (2010). Expression of TLR2, TLR4, and TLR9 in dermatomyositis and polymyositis. Clin. Rheumatol. *29*, 273–279. https://doi.org/10.1007/s10067-009-1316-7.
27. Li, Y., Wilson, H.L., and Kiss-Toth, E. (2017). Regulating STING in health and disease. J. Inflamm. *14*, 11. https://doi.org/10.1186/s12950-017-0159-2.
28. Motwani, M., Pesiridis, S., and Fitzgerald, K.A. (2019). DNA sensing by the cGAS-STING pathway in health and disease. Nat. Rev. Genet. *20*, 657–674. https://doi.org/10.1038/s41576-019-0151-1.
29. Pawaria, S., Sharma, S., Baum, R., Nündel, K., Busto, P., Gravallese, E.M., Fitzgerald, K.A., and Marshak-Rothstein, A. (2017). Taking the STING out of TLR-driven autoimmune diseases: good, bad, or indifferent? J. Leukoc. Biol. *101*, 121–126. https://doi.org/10.1189/jlb.3MR0316-115R.
30. Tornabene, T., Bourne, T., Raziuddin, S., and Ben-Amotz, A. (1985). Lipid and lipopolysaccharide constituents of cyanobacterium Spirulina platensis (Cyanophyceae, Nostocales). Mar. Ecol. Prog. Ser. *22*, 121–125.
31. Hoshino, K., Takeuchi, O., Kawai, T., Sanjo, H., Ogawa, T., Takeda, Y., Takeda, K., and Akira, S. (1999). Cutting edge: Toll-like receptor 4 (TLR4)-deficient mice are hyporesponsive to lipopolysaccharide: evidence for TLR4 as the Lps gene product. J. Immunol. *162*, 3749–3752.
32. Kawai, T., Takeuchi, O., Fujita, T., Inoue, J., Mühlradt, P.F., Sato, S., Hoshino, K., and Akira, S. (2001). Lipopolysaccharide stimulates the MyD88-independent pathway and results in activation of IFN-regulatory factor 3 and the expression of a subset of lipopolysaccharide-inducible genes. J. Immunol. *167*, 5887–5894.
33. Yamamoto, M., Sato, S., Hemmi, H., Uematsu, S., Hoshino, K., Kaisho, T., Takeuchi, O., Takeda, K., and Akira, S. (2003). TRAM is specifically involved in the Toll-like receptor 4–mediated MyD88-independent signaling pathway. Nat. Immunol. *4*, 1144–1150.
34. Akao, Y., Ebihara, T., Masuda, H., Saeki, Y., Akazawa, T., Hazeki, K., Hazeki, O., Matsumoto, M., and Seya, T. (2009). Enhancement of antitumor natural killer cell activation by orally administered Spirulina extract in mice. Cancer Sci. *100*, 1494–1501. https://doi.org/10.1111/j.1349-7006.2009.01188.x.
35. Yamamoto, M., Sato, S., Hemmi, H., Sanjo, H., Uematsu, S., Kaisho, T., Hoshino, K., Takeuchi, O., Kobayashi, M., Fujita, T., et al. (2002). Essential role for TIRAP in activation of the signalling cascade shared by TLR2 and TLR4. Nature *420*, 324–329. https://doi.org/10.1038/nature01182.
36. Sampath, P., Moideen, K., Ranganathan, U.D., and Bethunaickan, R. (2018). Monocyte subsets: phenotypes and function in tuberculosis infection. Front. Immunol. *9*, 1726. https://doi.org/10.3389/fimmu.2018.01726.
37. Kapellos, T.S., Bonaguro, L., Gemünd, I., Reusch, N., Saglam, A., Hinkley, E.R., and Schultze, J.L. (2019). Human monocyte subsets and phenotypes in major chronic inflammatory diseases. Front. Immunol. *10*, 2035. https://doi.org/10.3389/fimmu.2019.02035.
38. Menezes, S., Melandri, D., Anselmi, G., Perchet, T., Loschko, J., Dubrot, J., Patel, R., Gautier, E.L., Hugues, S., Longhi, M.P., et al. (2016). The heterogeneity of Ly6C(hi) monocytes controls their differentiation into iNOS(+) macrophages or monocyte-derived dendritic cells. Immunity *45*, 1205–1218. https://doi.org/10.1016/j.immuni.2016.12.001.
39. Sander, J., Schmidt, S.V., Cirovic, B., McGovern, N., Papantonopoulou, O., Hardt, A.L., Aschenbrenner, A.C., Kreer, C., Quast, T., Xu, A.M., et al. (2017). Cellular differentiation of human monocytes Is regulated by time-dependent interleukin-4 signaling and the transcriptional regulator NCOR2. Immunity *47*, 1051–1066.e12. https://doi.org/10.1016/j.immuni.2017.11.024.
40. Papadimitraki, E.D., Bertsias, G.K., and Boumpas, D.T. (2007). Toll like receptors and autoimmunity: a critical appraisal. J. Autoimmun. *29*, 310–318. Epub 2007 Oct 24. https://doi.org/10.1016/j.jaut.2007.09.001.
41. Bosisio, D., Polentarutti, N., Sironi, M., Bernasconi, S., Miyake, K., Webb, G.R., Martin, M.U., Mantovani, A., and Muzio, M. (2002). Stimulation of toll-like receptor 4 expression in human mononuclear phagocytes by interferon-gamma: a molecular basis for priming and synergism with bacterial lipopolysaccharide. Blood *99*, 3427–3431. https://doi.org/10.1182/blood.v99.9.3427.
42. Negishi, H., Fujita, Y., Yanai, H., Sakaguchi, S., Ouyang, X., Shinohara, M., Takayanagi, H., Ohba, Y., Taniguchi, T., and Honda, K. (2006). Evidence for licensing of IFN-gamma-induced IFN regulatory factor 1 transcription factor by MyD88 in Toll-like receptor-dependent gene induction program. Proc. Natl. Acad. Sci. USA *103*, 15136–15141. https://doi.org/10.1073/pnas.0607181103.
43. Shi, S., Nathan, C., Schnappinger, D., Drenkow, J., Fuortes, M., Block, E., Ding, A., Gingeras, T.R., Schoolnik, G., Akira, S., et al. (2003). MyD88 primes macrophages for full-scale activation by interferon-gamma yet mediates few responses to Mycobacterium tuberculosis. J. Exp. Med. *198*, 987–997. https://doi.org/10.1084/jem.20030603.
44. Kang, K., Bachu, M., Park, S.H., Kang, K., Bae, S., Park-Min, K.H., and Ivashkiv, L.B. (2019). IFN-γ selectively suppresses a subset of TLR4-activated genes and enhancers to potentiate macrophage activation. Nat. Commun. *10*, 3320. https://doi.org/10.1038/s41467-019-11147-3.
45. Torres-Ruiz, J., Carrillo-Vazquez, D.A., Padilla-Ortiz, D.M., Vazquez-Rodriguez, R., Nuñez-Alvarez, C., Juarez-Vega, G., and Gomez-Martin, D. (2020). TLR expression in peripheral monocyte subsets of patients with idiopathic inflammatory myopathies: association with clinical and immunological features. J. Transl. Med. *18*, 125. https://doi.org/10.1186/s12967-020-02290-3.
46. Brown, M., Davies, D.H., Skinner, M.A., Bowen, G., Hollingsworth, S.J., Mufti, G.J., Arrand, J.R., and Stacey, S.N. (1999). Antigen gene transfer to cultured human dendritic cells using recombinant avipoxvirus vectors. Cancer Gene Ther. *6*, 238–245. https://doi.org/10.1038/sj.cgt.7700014.
47. Yu, B., Wang, J., Suter, P.M., Russell, R.M., Grusak, M.A., Wang, Y., Wang, Z., Yin, S., and Tang, G. (2012). Spirulina is an effective dietary source of zeaxanthin to humans. Br. J. Nutr. *108*, 611–619. https://doi.org/10.1017/s0007114511005885.
48. Madhubalaji, C.K., Rashmi, V., Chauhan, V.S., Shylaja, M.D., and Sarada, R. (2019). Improvement of vitamin B(12) status with Spirulina supplementation in Wistar rats validated through functional and circulatory markers. J. Food Biochem. *43*, e13038. https://doi.org/10.1111/jfbc.13038.
49. Ranga Rao, A., Raghunath Reddy, R.L., Baskaran, V., Sarada, R., and Ravishankar, G.A. (2010). Characterization of microalgal carotenoids by mass spectrometry and their bioavailability and antioxidant properties elucidated in rat model. J. Agric. Food Chem. *58*, 8553–8559. https://doi.org/10.1021/jf101187k.
50. Wojcieszek, J., Witkoś, K., Ruzik, L., and Pawlak, K. (2016). Comparison of copper and zinc *in vitro* bioaccessibility from cyanobacteria rich in proteins and a synthetic supplement containing gluconate complexes: LC-MS mapping of bioaccessible copper complexes. Anal. Bioanal. Chem. *408*, 785–795. https://doi.org/10.1007/s00216-015-9162-8.
51. Li, Y., Bax, C., Patel, J., Vazquez, T., Ravishankar, A., Bashir, M.M., Grinnell, M., Diaz, D., and Werth, V.P. (2021). Plasma-derived DNA containing-extracellular vesicles induce STING-mediated proinflammatory responses in dermatomyositis. Theranostics *11*, 7144–7158. https://doi.org/10.7150/thno.59152.
52. Pillai, S., Nguyen, J., Johnson, J., Haura, E., Coppola, D., and Chellappan, S. (2015). Tank binding kinase 1 is a centrosome-associated kinase necessary for microtubule dynamics and mitosis. Nat. Commun. *6*, 10072. https://doi.org/10.1038/ncomms10072.

STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCES	SOURCE	IDENTIFIER
Antibodies		
APC anti-human Lineage cocktail/mouse host/20μL	Biolegend	Cat# 348803
PerCP-Cy5.5 anti-human HLA-DR/mouse host/5 μL	Biolegend	Cat# 307630; RRID: AB_893575
BUV395 anti-human CD14/mouse host/2μL	BD Biosciences	Cat# 563561; RRID: AB_2744288
BV480 anti-human CD16/mouse host/2μL	BD Biosciences	Cat# 566108; RRID: AB_2739510
PE-Cy7 anti-human CD11c/mouse host/5μL	Biolegend	Cat# 301608; RRID: AB_389350
BV711 anti-human CD123/mouse host/5μL	Biolegend	Cat# 306030; RRID: AB_2566353
BUV563 anti-human CD45/mouse host/2μL	BD biosciences	Cat# 748720; RRID: AB_2873124
BV605 anti-human CD3/mouse host/2μL	Biolegend	Cat# 317322; RRID: AB_11126166
BUV496 anti-human CD4/mouse host/2μL	BD Biosciences	Cat# 612936; RRID: AB_2870220
BV786 anti-human CD8/mouse host/2μL	BD Biosciences	Cat# 563823; RRID: AB_2687487
FITC anti-human IFNβ/mouse host/0.5μL	Pbl Assay science	Cat# 21400-3; RRID: AB_884211
BV650 anti-human IFNγ/mouse host/1.5μL	Biolegend	Cat# 502538; RRID: AB_2563608
APC-Cy7 anti-human TNFα/mouse host/2μL	Biolegend	Cat# 502944; RRID: AB_2562869
Critical commercial assays		
DUO ELISA kit- IFNβ	R&D systems	Cat# DY814
DUO ELISA kit- IFNγ	R&D systems	Cat# DY285B
DUO ELISA kit- TNFα	R&D systems	Cat# DY210
Other		
Spirulina	Sigma-Aldrich	Cat# 1619185
RPMI	Gibco	Cat# 11835-030
Tak242 (TLR4 inhibitor)	Cayman Chemicals	Cat# 13871
H151 (STING inhibitor)	Cayman Chemicals	Cat# 25857
MRT67307 (TBK inhibitor)	Cayman Chemicals	Cat# 19916
Ficoll-Paque PLUS gradient	GE Healthcare	Cat# 17144003
PBS	Corning	Cat# 21-031-CM
Brefeldin A	Biolegend	Cat# 420601

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Dr. Victoria P. Werth (werth@pennmedicine.upenn.edu).

Materials availability

This study did not generate new unique reagents.

Data and code availability

- This paper does not report original code.
- Data reported in this paper will be shared by the lead contact upon request.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.
- The authors confirm that the data supporting the findings of this study are available within the article and its supplementary materials.

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Patients

Patients from the DM database and controls from the Department of Dermatology at the Hospital of the University of Pennsylvania were recruited in accordance with an approved Institutional Review Board protocol (Protocol #808230). All subjects in the study signed an Informed Consent document before enrollment. There were total of 38 patients, predominately female with 33 female and 5 male, between the ages of 29 and 84. The race and ancestry consisted of 33 White/non-Hispanic, 3 Black/non-Hispanic, 1 White/Hispanic and 1 Asian/non-Hispanic. The patients demographics are listed in the "Demographics, Medications and DM classification table" (Table 1).

METHOD DETAILS

Spirulina preparation

100 mg pure Spirulina (Sigma-Aldrich) was dissolved in 50 mL of RPMI-1640 medium (Gibco).

RPMI-1640 medium with 0.2% BSA,[46] sonicated on high for 30 minutes, and centrifuged at 8500 rpm for 5 minutes. The Spirulina supernatant was collected, and the undissolved material was discarded. Spirulina stock concentration was 2 mg/mL Concentrations of Spirulina 0.3 mg/mL or 1 mg/mL were used for the experiments.

Determination of Spirulina concentration used in experiments

Concentrations were chosen based on Spirulina's bioavailability and attempts to mirror physiologic doses of Spirulina. As Spirulina is a plant-based protein consisting of hundreds of different components,[10] it is impossible to calculate the precise bioavailability. However, multiple different components of Spirulina have been found to have high bioavailability *in vivo*[47] and *in vitro*.[48–50] Using 4.7 liters of blood for the average adult, and assuming 100% of bioavailability of functional compounds of Spirulina in the average dose in store-bought green juice containing Spirulina (1,335 mg), the physiologic dose of Spirulina is 0.28 mg/mL.

Peripheral blood mononuclear cell (PBMC) isolation, stimulation with Spirulina, and culture

PBMCs were isolated from heparinized blood on an endotoxin-free Ficoll gradient (GE Healthcare) and resuspended in RPMI-1640 medium with 0.2% BSA. 1 x 10^6 million PBMCs were placed in each well. Spirulina-stimulated PBMCs were treated with 150μL or 500μL of Spirulina supernatant for a working concentration of Spirulina 0.3 mg/mL or 1 mg/mL, respectively. Wells were completed to 1mL with cell culture medium. To test the effects of TLR4 inhibitor (Tak242, Cayman Chemical, Ann Arbor, MI), STING inhibitor (H151, Cayman Chemical, Ann Arbor, MI), or TBK1 inhibitor (MRT67307, Cayman Chemical, Ann Arbor, MI) on stimulated versus unstimulated PBMCs, 1μg/mL TLR4 inhibitor, 1μM STING inhibitor, or 5μM TBK1 inhibitor were added 3 hours prior to Spirulina stimulation. Inhibitor concentrations for H-151, TAK242, and TBK1 were determined based on prior studies.[51,52] 15 hours after cells were treated, trypan blue staining was used to test cell viability.

Enzyme-linked immunosorbent assay (ELISA)

Cell-free supernatants were collected for ELISA. The concentrations of TNFα, IFNβ, and IFNγ were quantified in duplicate according to the manufacturer's protocol (R&D Systems, Minneapolis, MN). Because availability of supernatants limited testing of multiple cytokines by ELISA in some patients, different cytokine assays were tested using different patient cohorts in certain instances. However, all inhibitor/stimulation ELISAs for the same cytokine were performed using the same patient samples.

Flow cytometry

Stimulation of cells, staining, and acquisition of data

300μL or 1000μL Spirulina supernatant were added to PBMCs from DM patients, for a working concentration of Spirulina 0.3 mg/mL or 1 mg/mL, respectively. Wells were completed to 2mL with cell culture medium. 1μg/mLTLR4 inhibitor, 1μM STING inhibitor, or 5μM TBK1 inhibitor were added to all PBMCs 3 hours prior to Spirulina stimulation. PBMCs were stimulated with Spirulina for 15 hours at 37°C. Brefeldin A (1mg/mL) (Biolegend, San Diego, CA) was added to the cells 2 hours before staining and incubated at 37°C. A detailed staining protocol is discussed below. After staining, single-cell suspensions underwent flow cytometric analysis on FACS Symphony A3 Lite and were analyzed with FlowJo V10.7.1.

For lymphocyte, dendritic cell, and monocyte samples, 2 x10^6 cells were aliquoted into each FACS tube (Falcon Corning, Glendale, AZ). Samples were then pretreated with staining buffer (2% FBS in PBS), blocked with mouse IgG2b (Sigma Aldrich-I8765-10MG), and stained with surface antibodies (CD45, CD3, CD4, CD8 for lymphocytes and Lineage cocktail, HLA-DR, CD11c, CD123, and CB2R for dendritic cells and Lineage cocktail, HLA-DR, $CD14^+$, $CD16^+$ for monocytes) for 30 minutes at 4°C. Conjugated antibodies are detailed in key resources table. Cells were washed in PBS, fixed with Fixation buffer (Biolegend), 1x PBS solution with 4% paraformaldehyde and permeabilized with Intracellular Staining Permeabilization Wash Buffer (Biolegend), 10X to 1X in DI water and stained for intracellular cytokines (IFNγ, IFNβ, TNFα for lymphocytes, dendritic cells, and monocytes) for 20 minutes at room temperature. Cells were resuspended in 0.2mL PBS, and single-cell suspensions underwent flow cytometric analysis on a BD FACSymphony™ A3 Cell Analyzer (BD Biosciences, San Jose, CA) and were analyzed with FlowJo software (BD Biosciences). A total of 200,000 events were collected for analysis. Lymphocytes were identified by gating on leukocytes and identifying $CD45^+$ and, within that population, $CD3^+$ cells respectively. Within $CD3^+$ population, $CD4^+$ and $CD8^+$ were identified

by their respective positive quadrants (Figure S6A). Dendritic cells were identified by gating on leukocytes and identifying the HLA-DR$^+$ and Lineage negative subpopulation. This excluded CD3$^+$ T cells, CD14$^+$/CD16$^+$ monocytes/macrophages, CD16$^+$/CD56$^+$ NK cells, CD16$^+$ neutrophils, CD19$^+$/CD20$^+$ B cells. The mDCs and pDCs were identified by CD11c$^+$ and CD123$^+$, respectively (Figure S6B). The monocyte population was identified by gating and identifying the HLA-DR$^+$ and Lineage positive population. This population was further gated using CD14$^+$ to identify the monocyte population. This parent gate of CD14$^+$ was then gated on CD14$^+$ and CD16$^+$ to identify the monocyte subpopulations of classical Monocytes (CD14^{++}CD16$^-$), intermediate Monocytes (CD14^{++}CD16$^+$), and non-classical Monocytes (CD14$^+$CD16^{++}). The parent gate of CD14$^+$ was also further gated with CD11c$^+$ to identify the monocyte-derived dendritic cells (CD14$^+$CD11c$^+$) (Figure S6C).

Imaging mass cytometry

Spirulina history was elicited in a stepwise, controlled manner for all patients, using a flyer listing Spirulina-containing products to aid recall. Patients with a well-defined history of initiating first-time consumption of Spirulina-containing products in the immediate period preceding onset of new DM, i.e., Spirulina-associated DM, were selected (n=5). Controls were selected from patients with a new diagnosis of DM with no history of Spirulina ingestion (n=9). The average age for DM and Spirulina-associated DM patients was 53.4 and 52.8 years, respectively (p=0.77). DM patients were 87.5% female, and all Spirulina-associated DM patients were female. All DM patients were Caucasian, and Spirulina-associated DM patients were 80% Caucasian and 20% Asian. None of the patients in either group had positive myositis specific antibodies. The median time taking Spirulina before DM flare was 168 days (IQR=41-366).

Two 37 marker panels with a total of 53 unique metal conjugated antibodies were used to stain 4μm formalin fixed, paraffin embedded tissue sections. Images were then acquired on the Hyperion Imaging System (Fluidigm) and were processed as we have described previously (16). Cell cluster and patient FCS files were exported from histoCAT and imported to FlowJo Software™ Version 10.7.1. DM and Spirulina-associated DM cell counts and mean pixel intensities (MPI) were compared using the Mann-Whitney test with significance set at α=0.05.

QUANTIFICATION AND STATISTICAL ANALYSIS

One-way analysis of variance (ANOVA) with Tukey's multiple comparison tests and Bivariate comparisons using Students t-test were made with GraphPad Prism software (GraphPad 122 Software, Inc., La Jolla, CA, USA) and were used for ELISA and flow cytometry experiments. Comparisons of IMC-derived cell counts and intracellular cytokine staining were made using the Mann-Whitney U test, and correlations were analyzed using Pearson's R.