

# Immolina, a High–Molecular-Weight Polysaccharide Fraction of Spirulina, Enhances Chemokine Expression in Human Monocytic THP-1 Cells

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## ABSTRACT

**Introduction:** Spirulina (*Spirulina platensis*) is a dietary supplement valued for its immune-enhancing properties. We previously reported that the immunostimulatory effect of spirulina can be traced to a high–molecular-weight polysaccharide fraction. This fraction, labeled Immolina, activates nuclear factor kappa–B in human monocytic THP-1 cells and increases expression of proinflammatory cytokines.

**Objective:** To characterize further the immunostimulatory effects of Immolina on THP-1 cells, we evaluated its effect on genes encoding the chemokines interleukin (IL)–8, MCP-1, MIP-1 $\alpha$ , MIP-1 $\beta$ , IP-10, the cytokines tumor necrosis factor (TNF)– $\alpha$ , IL-1 $\beta$ , and the enzyme cyclo-oxygenase-2 (COX-2).

**Methods:** THP-1 cells were exposed to concentrations of Immolina ranging from 1 ng/mL to 100  $\mu$ g/mL and changes in gene expression were assessed by reverse transcriptase–polymerase chain reaction (RT-PCR). For comparison, THP-1 cells were activated with 1 ng/mL of TNF- $\alpha$ , 10 ng/mL of IL-1 $\beta$ , or 10 ng/mL of lipopolysaccharide using the same assay conditions. To assess the response of THP-1 cells to Immolina at the protein level, we probed culture supernatants using a cytokine array immunoblot assay.

**Results:** RT-PCR analysis revealed that Immolina dose-dependently increased the expression of all 5 chemokines tested as well as the expression of TNF- $\alpha$ , IL-1 $\beta$ , and COX-2. The cytokine array immunoblot assay revealed an increase in the chemokines IL-8 and MIP-1 $\beta$ . Thymidine uptake experiments verified that Immolina did not affect the viability and growth rate of THP-1 cells.

**Conclusions:** The results of the experiments demonstrate that Immolina activates THP-1 cells in a manner that is consistent with the recruitment of diverse populations of leukocytes in response to inflammatory and infectious signals.

## INTRODUCTION

**S**pirulina (*Spirulina platensis*) is a member of the blue–green alga family that is attracting considerable interest as a dietary supplement.<sup>1</sup> Since the early 1970s, spirulina has been used as a feed additive in aquaculture and poultry nutrition and has been found to have a positive effect on the health status of farm animals. These observations

led to numerous *in vitro* and *in vivo* studies in attempts to characterize the biologic effects of spirulina and its pharmacologic mechanisms of action.

Hayashi et al.<sup>2</sup> were among the first to report that mice on a spirulina diet showed an enhanced primary immune response to sheep red-blood cells. These investigators also noted that a water extract of the blue–green alga increased the proliferation of spleen cells in culture and enhanced interleukin-

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1 (IL-1) production by peritoneal macrophages. Leukocyte activating effects of spirulina were subsequently confirmed by other laboratories. Dietary spirulina was found to enhance humoral and cell-mediated immune functions in chickens as reflected in an increased phagocytic activity of macrophages and higher antibody titers.<sup>3,4</sup> Immunostimulatory effects have also been observed in human volunteers after oral administration of spirulina as reflected in an enhanced  $\gamma$ -interferon (IF- $\gamma$ ) production from peripheral blood mononuclear cells and increased natural killer (NK) cell cytotoxicity.<sup>5</sup> These experimental data provided scientific evidence to support the empirical observation that spirulina enhances the resistance to infections by bacteria, fungi, parasites, and viruses.<sup>1</sup>

The identity of the constituents accounting for the immunostimulatory effects has not yet been established. Polysaccharides of several herbal preparations are known for their immunomodulating and antitumor properties.<sup>6,7</sup> Recently, Pugh et al. isolated a high molecular weight polysaccharide preparation from *Spirulina platensis*.<sup>8</sup> This preparation, referred to as Immolina, has potent immunostimulatory activity as measured by nuclear factor kappa-B (NF- $\kappa$ B) activation and the induction of genes encoding the cytokines tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and IL-1 $\beta$  (IL-1 $\beta$ ). This observation added Immolina to the list of immunostimulatory polysaccharides isolated from various plants and fungi.<sup>7,9-11</sup>

The human THP-1 monocytic cell line is commonly used as a surrogate in studies of cells of the monocyte/macrophage system.<sup>12</sup> Here we show that Immolina dose-dependently induces genes encoding chemokines in THP-1 cells in addition to inducing the proinflammatory cytokines TNF- $\alpha$  and IL-1 $\beta$ , and the enzyme cyclo-oxygenase-2 (COX-2). These findings support the proposition that the immunostimulatory effects of spirulina can be attributed to the ability of its high molecular weight polysaccharide fraction to stimulate the expression of genes involved in immune and inflammatory processes.

## MATERIALS AND METHODS

### Materials

The bacterial endotoxin lipopolysaccharide (LPS from *Escherichia coli* 055:B5), TNF- $\alpha$  and IL-1 $\beta$  were purchased from Sigma Aldrich (St. Louis, MO). Human monocytic THP-1 cells were obtained from the American Type Culture Collections (ATCC). THP-1 cells were propagated in Roswell Park Memorial Institute (RPMI) 1640 supplemented with 10% fetal bovine serum (FBS; Gemini Bioproducts, Sacramento, CA).

### Preparation and characterization of Immolina

*Spirulina platensis* raw material was extracted twice with 50% ethanol at 70°C. Supernatants from both extractions were combined following centrifugation at 1500  $\times$  g. The ethanol concentration of the supernatant was adjusted to 75% by ad-

dition of 1 volume of cold ethanol. Following incubation at -20°C for several hours, precipitable material was collected by centrifugation at 1500  $\times$  g and washed with cold 95% ethanol. The final extract material, Immolina, was dried and represented a 16% yield of raw material dry weight.

### Stimulation of THP-1 cells with Immolina, TNF- $\alpha$ , IL-1 $\beta$ and LPS

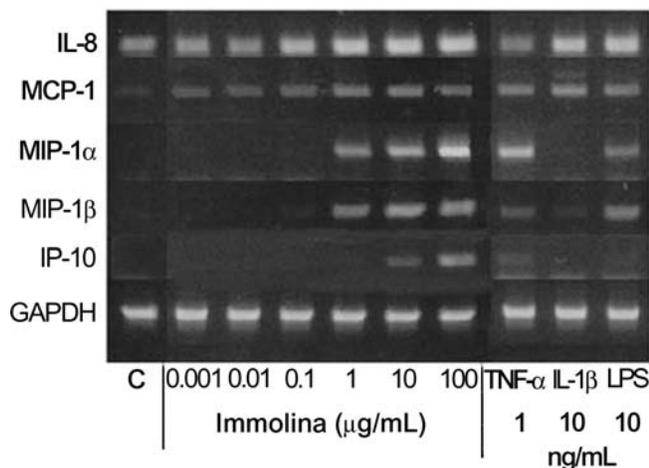
THP-1 cells ( $5 \times 10^5$  cells/well in an RPMI medium containing 10% FBS) were incubated with medium alone, Immolina ranging from 0.001 to 100  $\mu$ g/mL, 1 ng/mL of TNF- $\alpha$ , 10 ng/mL of IL-1 $\beta$ , or 20 ng/mL of LPS. Cells were incubated for 1 hour at 37°C in 5% CO<sub>2</sub>. The cell suspension was then centrifuged at 200  $\times$  g and the cell pellet was frozen at -70°C for subsequent ribonucleic acid (RNA) extraction with TRIzol (Invitrogen, Carlsbad, CA).

### Reverse transcriptase-polymerase chain reaction and real-time polymerase chain reaction analyses

A total cDNA library was synthesized using the Advantage reverse transcriptase-polymerase chain reaction (RT-PCR) kit (Clontech Laboratories, Palo Alto, CA) and Oligo (dT)<sub>18</sub> primer (Invitrogen). The resulting reverse transcriptase product was expanded using the SuperTaq Plus (Ambion, Austin, TX) PCR kit and specific primers for TNF- $\alpha$ , IL-1 $\beta$ , COX-2, macrophage inflammatory protein 1 $\alpha$  and 1 $\beta$  (MIP-1 $\alpha$  and MIP-1 $\beta$ ), mononuclear phagocyte chemoattractant protein-1 (MCP-1), IF- $\gamma$  inducible protein 10 (IP-10) and the housekeeping gene glycerol dehydrogenase (GAPDH). Two (2) mL of cDNA template were used in each PCR reaction. The PCR products were analyzed by electrophoresis on a 1.5% agarose gel containing ethidium bromide. For real-time PCR analysis, cDNA for the housekeeping gene 18s and IL-1 $\beta$  was amplified using primers from Invitrogen (Carlsbad, CA). The following TaqMan probes for 18s were used: TaqMan PCR Core Reagents Kit, Human 18s X03205-280F, Sequence: GCGGCTTTGGT-GACTCTAGATAA; Human 18s X03205-384R, Sequence: ACGGCGACTACCATCGAAAGT; and the TaqMan probe: Human 18s X03205-343T, Sequence: ATTCTGAACGTCT-GCCC. The following TaqMan probes for IL-1 $\beta$  were used: Human IL1  $\beta$  NM-000576-157F, Sequence: TTTGAAGCT-GATGGCCCTAAA, Human IL-1  $\beta$  NM-000576-274R; Sequence: GCCTGAAGCCCTTGCTTAGT; and the TaqMan probe: Human IL-1 $\beta$  NM-000576-232T, Sequence: CAGC-TACGAATCTC. TaqMan probes were obtained from Applied Biosystems (Foster City, CA). The ABI PRISM 7700 Sequence Detector was used at the following temperature and duration cycles: 50 2'-1, 95 10'-1, 95 15'', 60 1'-40.

### Cytokine array immunoblot assay

The presence of cytokines and chemokines in THP-1 cell culture supernatants was probed by immunohistochemistry using a RayBio Inflammation Antibody Array (RayBio,



**FIG. 1.** Reverse transcriptase–polymer chain reaction chemokine mRNA expression profiles for nonactivated and activated THP-1 cells. Cells were incubated with control medium alone (column 1) or with Immolina (columns 2–7) at doses ranging from 1 ng to 100  $\mu\text{g/mL}$ . For comparison, THP-1 cells were also incubated with tumor necrosis factor (TNF)- $\alpha$  (1 ng/mL, column 8), IL-1 $\beta$  (10 ng/mL, column 9) and LPS (10 ng/mL, column 10). Individual chemokine profiles were compared to the housekeeping gene glycerol dehydrogenase (GAPDH). Note that the response to Immolina was dose-dependent. Interleukin (IL)-8 and MCP-1 gene induction was detected at lower doses of Immolina than those for MIP-1 $\alpha$ , MIP-1 $\beta$  and IP-10.

Norcross, GA) membrane (RayBio Human Inflammation Antibody Array III). THP-1 cells ( $5 \times 10^5$  cells/well) were incubated in media alone (control), or in the presence of Immolina (100  $\mu\text{g/mL}$ ), IL-1 $\beta$  (10 ng/mL), and LPS (20 ng/mL), respectively, for 24 hours. Two (2) mL of supernatant were used for the immunoblot assay. Captured cytokines and chemokines were visualized by immunohistochemistry following the instruction of the manufacturer.

#### Determination of DNA synthesis by $^3\text{H}$ -thymidine uptake

THP-1 cells ( $2 \times 10^3/200 \mu\text{L}$ ) were plated with control medium alone or different concentrations of Immolina. Each well was labeled with 1  $\mu\text{Ci}$   $^3\text{H}$ -thymidine and the plate was incubated for 24 hours at 37°C, 5%  $\text{CO}_2$ . Cells were subsequently harvested on glass fiber filters using a Brandel (Brandel, Gaithersburg, MD) harvester. Radioactivity incorporated into DNA was determined using a Beckman multipurpose scintillation counter (Model LS 6500; Beckman Coulter, Fullerton, CA). Results were analyzed by Dunnett analysis of variance using JMP IN software (SAS Institute Inc., Cary, NC).

## RESULTS

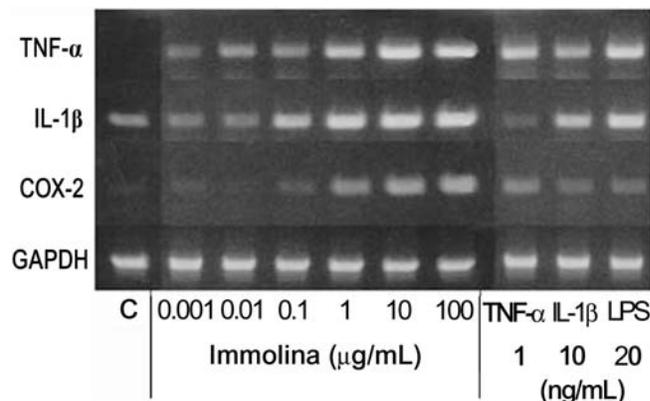
#### Effect of Immolina on chemokine mRNA levels

To determine the effect on chemokine expression, THP-1 cells were exposed to varying amounts of Immolina for 1

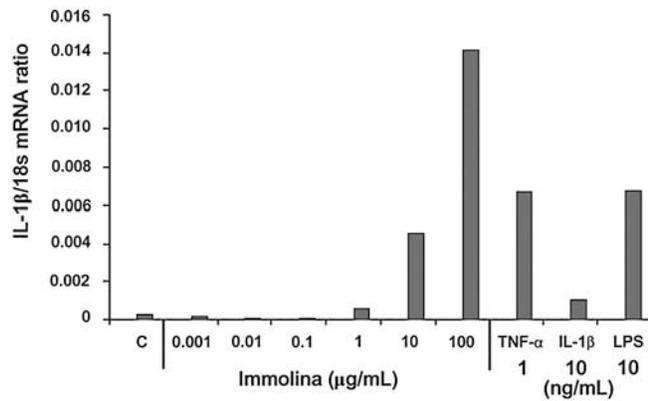
hour. For comparison of the response, THP-1 cells were also exposed to TNF- $\alpha$ , IL-1 $\beta$  and LPS, respectively. As shown in Figure 1, Immolina produced a dose-dependent increase in mRNA levels for each of the chemokines tested. In the case of IL-8 and MCP-1, detectable levels of mRNA were observed even at low doses of Immolina (Fig. 1, rows 1 and 2) while increases in mRNA levels for MIP-1 $\alpha$  and MIP-1 $\beta$  were seen only at higher doses (Fig. 1, rows 3 and 4). IP-10 was least responsive to Immolina with noticeable increases in mRNA levels detectable only at the highest doses (Fig. 1, row 5). The magnitude of the response of the chemokine IL-8 to 1 ng/mL of Immolina was comparable to that observed with 1 ng/mL of TNF- $\alpha$  but less than that of 10 ng/mL of IL-1 $\beta$  or 20 ng/mL of LPS (Fig. 1, columns 8–10).

#### Effect of Immolina on TNF- $\alpha$ , IL-1 $\beta$ and COX-2 mRNA levels

To determine the dose-dependence of the response of THP-1 cells to Immolina, mRNA levels of TNF- $\alpha$ , IL-1 $\beta$  and COX-2 were determined in cell extracts 1 hour after exposure to Immolina. As shown in Figure 2, Immolina produced a dose-dependent effect on mRNA levels of TNF- $\alpha$ , IL-1 $\beta$ , and COX-2. Compared to controls, concentrations of Immolina as low as 1 ng/mL induced detectable levels of TNF- $\alpha$  mRNA (Fig. 2, row 1). One-hundred ng/mL of Immolina was required to detect an increase in IL-1 $\beta$  mRNA. Real-time PCR analysis confirmed the dose-dependent induction of IL-1 $\beta$  and showed that 100 ng/mL of Immolina induced quantifiable amounts of transcripts (Fig. 3). Con-



**FIG. 2.** Reverse transcriptase–polymer chain reaction analysis of tumor necrosis factor (TNF)- $\alpha$ , interleukin (IL)-1 $\beta$  and cyclo-oxygenase (COX)-2 mRNA expression profiles in THP-1 cells activated with Immolina, TNF- $\alpha$ , IL-1 $\beta$ , and lipopolysaccharide (LPS). The mRNA expression profile in unstimulated THP-1 cells is shown in column 1. Columns 2–7 depict the dose-dependence of the response of cytokine genes in THP-1 cells exposed to Immolina. For comparison, columns 8–10 show the response of cytokine genes to the immunostimulants TNF- $\alpha$ , IL-1 $\beta$  and LPS. At 1  $\mu\text{g/mL}$ , Immolina produces a cytokine mRNA induction pattern in THP-1 cells that is similar to that seen with 10 ng/mL of LPS.



**FIG. 3.** Real-time polymerase chain reaction (PCR) analysis of interleukin (IL)-1 $\beta$  in THP-1 cells activated with Immolina, tumor necrosis factor (TNF)- $\alpha$ , IL-1 $\beta$  and lipopolysaccharide (LPS). Increased transcript levels of IL-1 $\beta$  were observed at  $\geq 1$   $\mu\text{g}/\text{mL}$  of Immolina.

centrations in the  $\mu\text{g}/\text{mL}$  range increased COX-2 expression (Fig. 2, row 3). The induction of TNF- $\alpha$ , IL-1 $\beta$ , and COX-2 mRNA levels in response to Immolina was weaker than the response to LPS (Fig. 2, column 10).

#### Cytokine array immunoblot assay

To determine whether the increases in chemokine expression can also be detected at the protein level, a membrane containing an array of 40 different capturing antibodies to proteins involved in inflammatory responses was incubated with supernatants of THP-1 cells stimulated with Immolina for 24 hours. Membranes spotted with capture antibodies to various cytokines and chemokines including TNF- $\alpha$ , IL-1 $\beta$ , IL-8, MIP-1 $\alpha$ , MIP-1 $\beta$ , MCP-1 and IP-10 were processed for immunohistochemistry. Figure 4 shows the results of the immunohistochemical staining of membranes incubated with supernatants from THP-1 cells grown in media alone (control, upper left panel), media containing IL-1 $\beta$  (lower left panel), media containing LPS (upper right panel), and media containing Immolina (lower right panel). Dots labeled 1 on each panel represent positive controls, dots labeled 2 represent IL-1 $\beta$ , dots labeled 3 represent IL-8 and those labeled 4 represent MIP-1 $\beta$ . As shown in Figure 4, supernatants of THP-1 cells stimulated with LPS and Immolina yielded strong staining of IL-8 and MIP-1 $\beta$  (Fig. 3, upper and lower right panels).

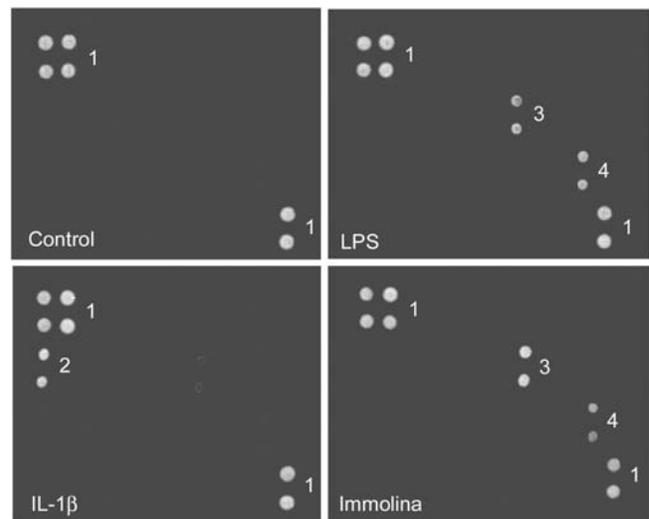
#### Viability/cytotoxicity assay

To assess whether Immolina displays any cytotoxicity or growth inhibition, THP-1 cells were exposed to concentrations ranging from 0.001  $\mu\text{g}/\text{mL}$  to 100  $\mu\text{g}/\text{mL}$  for 24 hours and DNA synthesis was determined based on [ $^3\text{H}$ ]-thymidine uptake. Immolina did not affect the viability nor the rate of proliferation of THP-1 cells at any of the doses tested

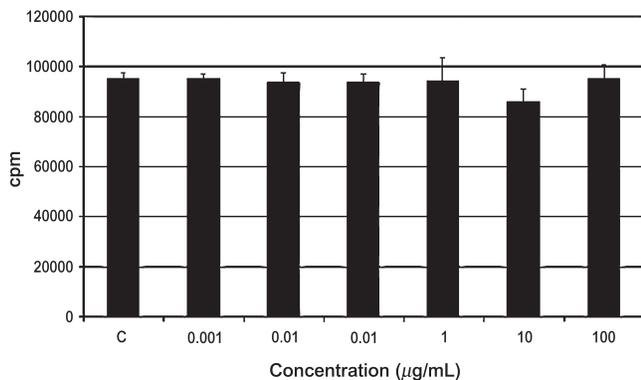
(Fig. 5). Each bar represents the mean  $\pm 1$  standard deviation of 9 replicates;  $p > 0.05$ .

## DISCUSSION

Immunostimulants enhance host defense mechanisms against bacterial and viral infections, and strengthen host immunity to tumors. Their mode of action relies on nonspecific activation of cells of the immune system, in particular cells of the monocyte/macrophage system.<sup>13</sup> Among the diverse classes of molecules that have been shown to act as immunostimulants are polysaccharides of microbial origin and plant origin.<sup>2,6,10,11,14</sup> Recently, a polysaccharide fraction isolated from the blue-green alga spirulina and referred to as Immolina was found to activate NF- $\kappa\text{B}$  in THP-1 cells and increase mRNA levels of TNF- $\alpha$  and IL-1 $\beta$ . These two cytokines are key mediators and regulators of innate immunity. The results of the present study demonstrate that Im-



**FIG. 4.** Cytokine and chemokine protein production induced by Immolina was probed using the RayBiotech (Norcross, GA) human inflammation antibody array III assay. This assay allows the simultaneous detection of multiple cytokines and chemokines in supernatants of THP-1 cells exposed to immunostimulants. THP-1 cells were stimulated with interleukin (IL)-1 $\beta$  (10 ng/mL), lipopolysaccharide (LPS) (10 ng/mL) and Immolina (100  $\mu\text{g}/\text{mL}$ ). Membranes spotted with antibodies to various cytokines and chemokines were incubated with supernatants of unstimulated and stimulated THP-1 cells. The panel in the upper left hand corner shows the results of a control experiment in which supernatant of THP-1 cells incubated with only media alone were probed. The quadruplet of dots in the upper left and the duplet of dots in the lower right represent positive standards (labeled 1 in each of the figures). IL-1 $\beta$  is a potent autoinducer of IL-1 $\beta$  (labeled 2) but had no detectable effect on inflammatory protein production. LPS (10 ng/mL) evoked a significant increase in IL-8 (marked 3) and MIP-1 $\beta$  (marked 4). Like LPS, Immolina (100  $\mu\text{g}/\text{mL}$ ) induced the secretion of IL-8 and MIP-1 $\beta$  by THP-1 cells into the media.



**FIG. 5.** [<sup>3</sup>H] thymidine uptake experiments with THP-1 cells exposed to the concentration range used in this study. No significant differences in thymidine uptake were detected at any of the doses tested in the experiments ( $p > 0.05$ ). cpm, counts per minute.

molina induces several genes encoding chemokines involved in inflammatory responses and the enzyme COX-2 in THP-1 cells. These cells share many characteristics with cells of the monocyte/macrophage system<sup>12</sup> and are commonly used as surrogates in studies of innate immune responses. The results of this study show that Immolina potently and dose-dependently increases the expression of genes encoding the chemokines IL-8, MCP-1, MIP-1 $\alpha$ , MIP-1 $\beta$ , and IP-10. This family of chemotactic cytokines stimulates leukocyte movement and regulates the migration of leukocytes from blood to tissues. In addition, this study documents that Immolina increases the expression of the gene encoding COX-2 suggesting that this preparation may stimulate the production of prostaglandins. There is a notable discrepancy between the readily detected levels of chemokine mRNA levels and the failure to detect some of the chemokines at the protein level on the cytokine array membrane. This observation may indicate a lack of a correlation between mRNA levels and their corresponding proteins. Alternatively, this finding may merely be due to a lack of sensitivity of the antibodies used as capturing and detection antibodies in these experiments.

Chemokines are a family of structurally related proteins that play a crucial role in shaping host inflammatory and immune responses.<sup>15</sup> Inducible chemokines regulate the recruitment of leukocytes for amplification of the host response to infectious and inflammatory signals.<sup>16</sup> Individual chemokines preferentially target different populations of leukocytes.<sup>15</sup> For example, IL-8 directs the recruitment of neutrophils. In contrast, MIP-1 $\alpha$  and MIP-1 $\beta$  induce the influx of NK cells, macrophages, and immature dendritic cells while IP-10 guides activated T-cells into tissues. Our observation that Immolina increases the expression of multiple chemokine genes suggests that it may enable cells of the monocyte/macrophage system to attract different populations of leukocytes to sites of inflammation. The immunoblot assay showed that chemokine production by THP-1

cells in response to Immolina is particularly pronounced for IL-8 and MIP-1 $\beta$  (Fig. 4). Neutrophils are the primary target of IL-8 and respond to this mediator by chemotaxis, enhanced exocytosis of their vesicles and granules, and release of reactive oxygen intermediates. Stimulation of IL-8 production may be a mechanism by which Immolina supports the host in recruiting a greater number of peripheral mononuclear cells to inflammatory foci. Pathogen-induced release of MIP-1 $\alpha$  and MIP-1 $\beta$  has been shown to be vital for the recruitment of NK cells and may account for the increased NK cell activity that has been observed in human volunteers after oral administration of spirulina.<sup>5</sup> Previous reports have shown that spirulina activates T-cells and NK cells. These cells express receptors for IP-10 that are thought to be important for their recruitment into tissues.<sup>17</sup> With each of the chemokines preferentially targeting a different cell type, the finding that Immolina induces genes encoding several different chemokines points to a mechanism by which it may mediate host resistance to infections.

The results of this study confirm our previous observation that Immolina induces TNF- $\alpha$  and IL-1 $\beta$  expression. The pattern of cytokine and chemokine induction by Immolina is similar to that of LPS, one of the most powerful immunostimulants. Given that the expression of cytokines and chemokines is stimulated by signals that interact with various pattern recognition receptors such as members of the Toll family, it will be interesting to determine whether Immolina and LPS produce their immunostimulatory effects by similar receptor mechanisms. The observation that Immolina induces COX-2 in THP-1 cells suggests that it may increase the synthesis of arachidonic acid metabolites such as prostaglandin E<sub>2</sub>. Arachidonic acid metabolites are potent mediators of inflammation and may participate in protective responses.<sup>18</sup> Immolina may therefore be suited to restore depressed prostaglandin production in immunocompromised patients to functional levels. The observation that THP-1 cells respond to Immolina with an increased expression of COX-2 is in contrast to the potent and highly selective COX-2 inhibitory effect of phycocyanin, a component of spirulina.<sup>19</sup> Interestingly, Romay et al. reported that phycocyanin administered to mice 1 hour before LPS, dose-dependently inhibited the induction of TNF- $\alpha$  levels in serum.<sup>20</sup> Thus, unlike Immolina, spirulina has both pro- and anti-inflammatory properties. It will be of considerable interest to explore whether these differences are reflected in differences in the therapeutic properties of Immolina compared to spirulina. Several toxicologic studies have established the safety of spirulina products in laboratory animals.<sup>21–23</sup> In 2003, the U.S. Food and Drug Administration labeled a preparation derived from spirulina as a substance generally recognized as safe (GRAS; [www.fsan.da.gov/~rdb/opa-gras.html](http://www.fsan.da.gov/~rdb/opa-gras.html)). The results of our [<sup>3</sup>H] thymidine uptake experiments document that Immolina does not display any toxicity toward THP-1 cells as reflected in the lack of an effect on their viability and rate of proliferation (Fig. 5).

## CONCLUSIONS

Polysaccharides are increasingly recognized for their non-specific immunomodulatory effects and are being explored for therapeutic potential.<sup>6</sup> A common mechanism underlying their biologic effects is the activation of cells of monocyte/macrophage system via regulation of proinflammatory mediators in the cytokine network. Immolina appears to share this mode of action with a number of polysaccharides isolated from various plant sources.<sup>24,25</sup> In addition to their role as attractants of blood leukocytes to sites of infection and inflammation, chemokines have attracted attention for their ability to block HIV infections.<sup>26</sup> In view of its remarkable safety record, spirulina has recently been proposed for the prevention and treatment of HIV infections.<sup>27</sup> The results of our study suggest that Immolina, because of its potent immunostimulatory effects, may be a valuable supplement for the treatment of HIV infections. Further characterization of Immolina may provide a well-defined preparation for the prevention and treatment of bacterial and viral infections.

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