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Modulation of immune cell proliferation and chemotaxis towards CC chemokine ligand (CCL)-21 and CXC chemokine ligand (CXCL)-12 in undenatured whey protein-treated mice

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Abstract

Whey protein concentrates (WPCs) enhance innate mucosal immunity during early life and have a protective role in some immune disorders. To further elucidate the potential benefits of this protein, the present study investigated the effect of dietary supplementation with WPCs on blood parameters, plasma cytokine profiles, and immune cell proliferation and chemotaxis. A total of 45 male mice were equally distributed into three experimental groups and treated daily for 21 days as follows: group I was a control group that was orally supplemented with distilled water, group II was orally supplemented with undenatured WP (100 mg/kg body weight), and group III was orally supplemented with bovine serum albumin (100 mg/kg body weight). We found that the plasma cytokine levels of interleukin (IL)-1α, IL-1β, IL-10 and tumor necrosis factor-α and the levels of reactive oxygen species, cholesterol, triglycerides and the lipid profile were significantly decreased in the WP-treated group compared to the control group. In contrast, the levels of IL-2, IL-4, IL-7, IL-8 and glutathione were significantly elevated, and consequently, the ability of peripheral blood mononuclear cells to proliferate in response to stimulation with different antigens was significantly increased in the WP-treated group. Moreover, the *in vitro* chemotaxis of B, T and bone-marrow-derived dendritic cells toward CC chemokine ligand-21 and CXC chemokine ligand-12 was significantly increased, by twofold, in WP-treated mice compared to the control group. Taken together, our data reveal the benefits of WP supplementation in enhancing immune cell proliferation and migration to the secondary lymphoid organs.

Keywords: Chemotaxis; Cytokines; Free radicals; Glutathione; Lipid profile; Whey proteins

1. Introduction

Secondary lymphoid tissues are the organs in which B and T cells associate with antigen-presenting cells (APCs) to initiate an adaptive immune response. These tissues are also the sites in which naive CD4 and CD8 T cells encounter foreign antigens (Ags) presented by professional APCs and are activated, differentiating into effector lymphocytes [1]. Specifically, secondary lymphoid organs, such as the spleen, peripheral lymph nodes (PLNs), mesenteric lymph nodes and Peyer's patches, are sites of intense lymphocyte trafficking. The tissue-specific homing of blood-borne naive lymphocytes into secondary lymphoid tissues is critical to ensure frequent and efficient encounters between APCs and antigen-specific T cells [2,3]. Effective immune responses depend on an array of soluble mediators that are collectively called cytokines [4]. For instance, activated cytotoxic T lymphocytes (CTLs) secrete various cytokines, such as interferon gamma (IFN-γ) and tumor necrosis factor alpha (TNF-α), which enhance antigen presentation and mediate antipathogenic effects. Previous reports have shown that various cytokines, such as interleukin (IL)-2, or IFN-γ-producing CD4 T cells are required for the generation of effective CTL immunity against infection and cancer [5]. Moreover, type I natural killer T cells have the remarkable ability to produce both Th1 (e.g., IFN-γ) and Th2 (e.g., IL-4 and IL-10) cytokines upon stimulation and can subsequently help activate other immune cells, such as T cells, natural killer cells or dendritic cells (DCs) [6].

Chemokines are small chemoattractant cytokines that bind to specific G-protein-coupled receptors present on the plasma membranes of target cells [7]. Chemokines play a central role in lymphocyte trafficking and homing in primary and secondary lymphoid organs and inflamed tissues [8]. They provide specific signaling to leukocytes for extravasation from the blood and direct

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locomotion as well as microenvironmental homing of leukocytes within tissues [9], all of which are critical for the accumulation of immune cells at sites of infection. Chemokines and their receptors are important regulators of cell migration to specific anatomic areas, such as lymph nodes and sites of infection, and thus have an important effect on cell function [10]. Therefore, the chemokine receptors are crucial for homing to secondary lymphoid organs and, subsequently, for Ag recognition [11]. Chemokines play a critical role in the chemotaxis of B cells. In particular, CC chemokine ligand (CCL)-21 participates in the recruitment of naive T cells, DCs and B cells to the extrafollicular area in secondary lymphoid organs [12,13]. These chemokines are produced by cells scattered throughout the extrafollicular area and act through chemokine receptor7 (CCR7), which is specifically expressed on activated T and B cells and mature DCs [14]. The differentiation stage and the engagement of the B cell receptor modulate migration in response to chemokines such as CXC chemokine ligand (CXCL)-12 [15].

Antioxidants play a vital role in maintaining immunity and protection against free radical damage in the human body. Whey proteins (WPs) represent a heterogeneous group of proteins (β-lactoglobulin, α-lactalbumin, serum albumin and immunoglobulins). Indeed, recently published data have suggested that WP has antioxidant activity, probably owing to the abundance of cysteine in WP or the presence of glutamylcysteine groups, which are also found in other food proteins. Therefore, WP may be a therapeutic tool for oxidative-stress-associated diseases [16]. Previous studies have revealed that oral administration of an undenatured cysteine-rich WP isolate increases glutathione (GSH) levels in several GSH-deficient patient groups, including those patients with advanced human immunodeficiency virus (HIV) infection [17]. In addition, *in vitro* and *in vivo* studies have demonstrated a clear modulation of immune functions by several WP-derived products [18,19]. WPs stimulate splenocyte proliferation, increase IL-1 production by macrophages and increase GSH production [20]. Moreover, whey peptides possess immunomodulatory activities, such as stimulating lymphocytes, an increase in phagocytosis and the secretion of immunoglobulin A by Peyer's patches [21]. Recently, it has been reported that WP has immunomodulatory properties and the potential to increase host defense [22] and anticancer effects [23]. To further elucidate the potential benefits of this protein, the present study investigated the impact of dietary supplementation with WP on blood parameters, plasma cytokine profiles, and immune cell proliferation and chemotaxis.

2. Materials and methods

2.1. Preparation of WPs

Raw camel milk was collected from healthy female camels (three camel breeds: Majaheim, Maghateer and Soffer) from the Riyadh area, Saudi Arabia, and was then centrifuged to remove the cream. The obtained skim milk was acidified to pH 4.3 using 1 N HCl at room temperature and centrifuged at 10,000*g* for 10 min to precipitate casein. The resultant whey, which contains the WPs, was saturated with ammonium sulfate to a final saturation of 80% to precipitate the WPs. The precipitated WPs were dialyzed against 20 vol of distilled water for 48 h using a molecular-porous membrane with molecular weight cutoff of 6000–8000 kDa. The dialysate containing undenatured WPs was freeze-dried and refrigerated until use.

2.2. Animals and experimental design

A total of 45 sexually mature 12-week-old male Swiss Webster (SW) mice, weighing 25–30 g each, were obtained from the Central Animal House of the Faculty of Pharmacy at King Saud University. All animal procedures were in accordance with the standards set forth in the *Guidelines for the Care and Use of Experimental Animals* by the Committee for the Purpose of Control and Supervision of Experiments on Animals and the National Institutes of Health. The study protocol was approved by the Animal Ethics Committee of the Zoology Department, College of Science, King Saud University. All animals were allowed to acclimatize in metal cages inside a well-ventilated room for 2 weeks prior to the experiment. Animals were maintained under standard laboratory conditions (temperature 23°C, relative humidity 60%–70% and a 12-h light/dark cycle) and were fed a diet of standard commercial pellets and given water *ad libitum*. Animals were distributed into three experimental groups (n=15/group): group I was a control group that was orally supplemented with distilled water (250 μl/mouse/day for 21 days through oral gavage), group II was orally supplemented with undenatured WP (100 mg/kg body weight dissolved in 250 μl/day for 21 days through oral gavage), and group III was orally supplemented with bovine serum albumin (BSA) (100 mg/kg body weight dissolved in 250 μl/day for 21 days through oral gavage). Therefore, the supplemented volume for the three groups was constant and did not exceed 250 μl per dosage.

2.3. Quantification of blood biochemical parameters

Animals were anesthetized using pentobarbital (60 mg/kg body weight), and whole blood was collected from the abdominal aorta and transferred into heparinized tubes immediately. Blood was then centrifuged at 4000*g* for 10 min using bench top centrifuge (MSE Minor, England) to remove red blood cells and recover plasma. Plasma samples were separated and were collected using dry Pasteur pipette and stored in the refrigerator for analysis. All analysis was completed within 24 h of samples collection. Blood glucose levels were measured using the glucose oxidase method using BioMerieux kits (France) according to the manufacturer's instructions, and protein levels were measured using the cupric ion reaction [24]. The intensity of the coloration was measured using a UV/visible Model 80-2106-00 spectrophotometer at 546 nm (Pharmacia Biotech, Cambridge, England).

Total plasma GSH concentrations were measured using high-performance liquid chromatography using fluorescence detection, as previously described [25]. Reactive oxygen species (ROS) levels were determined using 2,7-dichlorodihydrofluorescein diacetate (Beyotime Institute of Biotechnology, Haimen, China).

Lipid profiles were determined colorimetrically using BioMerieux kits and a standard assay method. Cholesterol levels were evaluated using the cholesterol esterase method [26]. Triglycerides were measured using the lipase method [27]. In the high-density lipoprotein (HDL) assay, performed according to Lopez-Virella et al. [28], low-density lipoprotein (LDL) and chylomicrons were precipitated using phosphotungstic acid. The amount of cholesterol bound to HDLs was determined using the cholesterol oxidase method and the phosphotungstate-magnesium salt method using a Cholesterol E-Test Kit (Wako, Osaka, Japan). The insulin level in plasma was estimated using an insulin radioimmunoassay kit (Huaxi Institute of Diabetic Technology, Chengdu, Sichuan Province, China).

2.4. Determination of plasma cytokine levels

The determination of the plasma cytokine profiles was performed using samples that had been stored at −80°C. Plasma cytokine levels (IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-7, IL-8, IL-10, IL-12 and TNF-α) were determined using enzyme-linked immunosorbent assays with mouse cytokine assay kits (R&D Systems) according to the manufacturer's instructions.

2.5. Total lymphocyte and B and T cell isolation

Lymphocytes were isolated from the spleens and PLNs of 12-week-old male mice. Lymph nodes and spleens were homogenized using 40-μm cell strainers (BD Falcon, Bedford, MA, USA). Red blood cells from spleens were osmotically lysed using ACK lysing buffer (Invitrogen) buffer. Cells were washed with phosphate-buffered saline (PBS), counted using the trypan blue exclusion test and cultured in R-10 culture medium (complete RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, 1 mM sodium pyruvate and 50 μM 2-mercaptoethanol). Splenic B cells were isolated by negative depletion using biotinylated antibodies against CD4, CD8, GR-1 and CDllc using Dynabeads M-280 Streptavidin (Invitrogen) as previously described [29]. Splenic T cells were also isolated using negative selection columns. The purity of B and T cells was assessed using flow cytometry and was greater than 95%. Cells were cultured in R-10 medium.

2.6. Preparation of bone-marrow-derived DCs

Bone-marrow-derived DCs (BM-DCs) were prepared as described previously [30], with slight modifications. BM cell suspensions were obtained from femurs and tibias. A total of 5×10^6 cells/ml was plated in six-well plates and left to adhere *in vitro* for 6 h in serum-free RPMI. A total of 5 ng/ml GM-CSF (Peprotech) was added for 10–14 days to 1×10^7 nonadherent cells/3 ml to induce DC differentiation. The culture medium was renewed every 3 days. Cell purity was assessed using flow cytometry and was greater than 96.5% $CD11c^+$ cells. In some experiments, BM-DCs were activated overnight using 1 μg/ml lipopolysaccharide (LPS) (*Escherichia coli*, Sigma).

2.7. Antibodies and flow cytometry

Lymphocytes from PLNs, spleens and blood as well as purified cell populations (1×10^6 cells per 50 μl PBS) were blocked with purified CD16/CD32 FccII/III mAb to prevent nonspecific binding. Subsequently, cells were stained with mAbs and analyzed using a FACSCalibur (BD, Franklin Lakes, NJ, USA). Antibodies against mouse CD11b,

CD11c, CD3, CD4, CD8a and B220 were purchased from BD Pharmingen. Anti-mouse CCR7, CCL-21 and CXCL-12 were purchased from R&D Systems.

2.8 In vitro chemotaxis assay

The chemotaxis of B and T cells and activated BM-DC populations was measured using migration through a polycarbonate filter with a 5-μm pore size in 24-well transwell chambers (Corning Costar, Cambridge, MA, USA), as previously described [31]. Briefly, 1×10^5 B or T cells or DCs in 100 μl of prewarmed migration medium (RPMI 1640 containing 10 mM HEPES and 1% FBS) were added to the upper chamber. A total of 600 μl of migration medium containing 250 ng/ml CCL-21 or CXCL-12 or medium alone as a control for spontaneous migration was added to the lower chamber, and the cells were incubated for 3 h at 37°C. Cells that transmigrated to the bottom chamber were collected and counted using flow cytometry (FACSCalibur, BD, Franklin Lakes, NJ, USA) for a fixed time period of 60 s. The mean number of spontaneously migrated cells was subtracted from the total number of migrated cells. The results are shown as the percentage of specific migration±standard error of the mean (S.E.M.).

2.9. Statistical analysis

Data were first tested for normality (using Anderson–Darling test) and for variances homogeneity prior to any further statistical analysis. Data were normally distributed and were expressed as the mean±S.E.M. Statistical differences between groups were analyzed using a one-way analysis of variance (for more than two groups) followed by Tukey's posttest using SPSS software, version 17. Differences were considered statistically significant at $P<.05$.

3. Results

3.1. Effect of the oral administration of WP on blood biochemical parameters

We monitored changes in blood parameters and leucocyte counts in all animal groups throughout the experimental period. We observed a significant decrease in the levels of the total lipid profile (cholesterol, triglycerides, HDL and LDL) and free radicals (hydroperoxide and ROS) in the WP-treated group compared with the control and BSA-treated groups. In contrast, a significant increase in the GSH level and white blood cell (WBC) count was observed in the WP-treated group compared to the control and BSA-treated groups (Table 1). Nevertheless, the administration of WP did not affect the levels of glucose, insulin, creatinine, alanine aminotransferase (ALT), aspartate aminotransferase (AST), total protein or hemoglobin (Hb), or the hematocrit value. Moreover, there were no differences in the body weight as well as in food intake among groups (data not shown).

Table 1
Effect of WP supplementation on blood parameters and the lipid profile

Parameters	Control	WP	BSA
Blood glucose (mg/dl)	142±11.7	131±10.2	136±9.6
Insulin (ng/ml)	2.4±0.1	2.12±0.15	2.3±0.19
ALT (U/ml)	45±3.9	48±4.2	43±4.1
AST (U/ml)	41±4.2	38±3.4	42±3.75
Creatinine (dg/ml)	3±0.2	3.3±0.23	5.6±0.49***
Total protein (g/100 ml)	5.6±0.5	5.75±0.52	6.1±0.45
Cholesterol (mg/100 ml)	74±6.7	61±5.8 *,**	76.5±7.1
Triglycerides (mg/dl)	34±2.4	23±1.9 *,**	38.6±3.4
HDL (mg/100 ml)	16.5±1.35	10.1±0.9 *,**	18.8±1.7
LDL (mg/100 ml)	35±2.9	22±2.1*,**	37.5±3.4
Hydroperoxide (mg/100 ml)	22.4±1.7	13.3±1.1*,**	24.2±2.1
GSH (μmol/ml)	112±5.7	149±7.9 *,**	118±6.8
WBC count ($\times10^3/mm^3$)	9.3±0.9	13.5±1.2 *,**	10.1±0.99
Lymphocyte count ($\times10^3/mm^3$)	5.8±0.45	7.9±0.38*	7.1±0.65
Neutrophil count ($\times10^3/mm^3$)	3.7±0.4	4±0.33	3.8±0.36
ROS level in WBCs (nmol/ml)	45±3.6	32±2.7*,**	47.4±4.1
RBC count ($\times10^3/mm^3$)	5.9±0.49	6.1±0.55	6.3±0.59
Hb (g/dl)	14±1.1	14.4±1.3	13.8±1.25
Hematocrit value (%)	34.9±3.1	35.3±3.3	35.1±2.9

Blood biochemical parameters were measured in the three groups of mice, and the results are presented as the mean±S.E.M.
*$P<.05$, WP vs. control; **$P<.05$, WP vs. BSA; ***$P<.05$, BSA vs. control (ANOVA with Tukey's posttest).

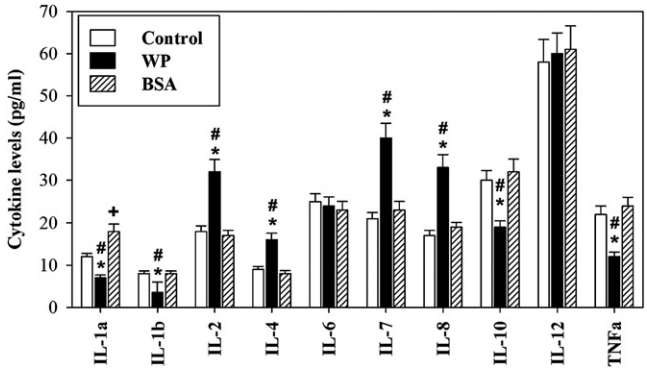

Fig. 1. Plasma cytokine profile. Plasma cytokine levels were determined in the three groups of mice ($n=8$). Results are expressed as the mean level of each cytokine±S.E.M.. *$P<.05$, WP vs. control; #$P<.05$, WP vs. BSA; +$P<.05$, BSA vs. control.

3.2. Alterations in the plasma levels of different cytokines

Because cytokines are secreted by specific cells of the immune system, carry signals locally between cells and are critical for the development and function of both the innate and the adaptive immune responses, we monitored alterations in the plasma levels of different cytokines that control immune cell functions after the oral administration of WP. WP-treated mice showed significant decreases in the levels of circulating IL-1α, IL-1β, IL-10 and TNF-α compared to the control and BSA-treated groups. Significant increases in the levels of IL-2, IL-4, IL-7 and IL-8 were clearly observed in the WP-treated group compared to the control and BSA-treated groups (Fig. 1). However, no significant changes were detected in the levels of IL-6 and IL-12 among the different groups.

3.3. The administration of WP enhances mitogen-mediated cell proliferation

Because WP-treated mice showed increases in the levels of IL-2, IL-4, IL-7 and IL-8 (cytokines favoring lymphocyte proliferation), we monitored peripheral blood mononuclear cell (PBMC) proliferation following stimulation with different mitogens using a carboxyfluorescein diacetate succinimidyl ester (CFSE) dilution assay. PBMCs were labeled with CFSE; stimulated using Con A, PHA, LPS, anti-CD3/CD28 mAbs or sIgM+sIL-4; and then cultured for 6 days prior to flow cytometry analysis. As shown in Fig. 2A, the plots were gated on lymphocytes according to the forward and side scatter and then on viable cells to exclude dead cells. The percentages of CFSE-lo (proliferating cells) and CFSE-high (nonproliferating cells) cells within the lymphocyte population are presented as histograms. In one representative experiment, we found that the percentage of lymphocyte proliferation following Con A stimulation was 42% in the control mice and 38% in BSA-treated mice and was increased to 83% in the WP-treated mice (Fig. 2A). Data from eight separate experiments (eight animals were randomly selected from each group) revealed that the percentage of proliferating lymphocytes following different stimulations was significantly increased in the WP-treated mice compared to the control and BSA-treated mice (Fig. 2B).

3.4. The administration of WP increases B cell chemotaxis to CCL-21 and CXCL-12

We assessed the chemotactic response of splenic B cells to CCL-21 and CXCL-12. After chemotaxis assays, input B cells, B cells that migrated to medium without chemokines and B cells that migrated to medium with CCL-21 or CXCL-12 were stained with B220 and CD3.

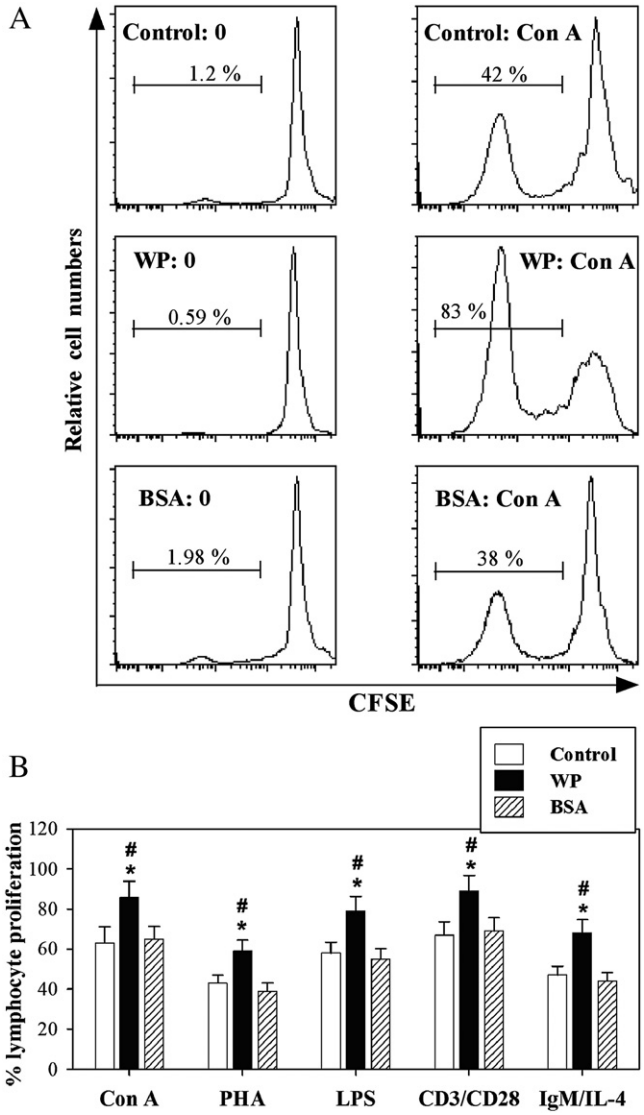

Fig. 2. Effect of WP on mitogen-induced PBMC proliferation. The ability of PBMCs to proliferate in response to mitogen stimulation was evaluated using the CFSE assay and flow cytometry. Histograms represent the results of the CFSE dilution assay, in which viable PBMCs were gated for control and WP- and BSA-treated mice. The mean of the left side of the histograms represents the percentage of CFSE-lo cells, whereas the mean of the right side of the histograms represents the percentage of CFSE-high cells within the PBMC populations. Histograms from one representative experiment (A) are shown for unstimulated (left column) and Con-A-stimulated (right column) cells. (B) Data from eight mice in each group are expressed as the percentage of cell proliferation ±S.E.M. in control (open bars), WP-treated (black bars) and BSA-treated (hatched bars) mice. $^{*}P<.05$, WP vs. control; $^{\#}P<.05$, WP vs. BSA; $^{+}P<.05$, BSA vs. control.

The cells were counted for 60 s using flow cytometry, and the number of $B220^{+}$ B cells that migrated to medium with or without CCL-21 and CXCL-12 was divided by the number of input B cells to determine the percentage of B cell chemotaxis. The percentage of B cells that migrated to the medium was subtracted from the percentage of B cells that migrated to chemokines to determine the number of cells that specifically migrated to chemokines. One representative experiment for B cell chemotaxis using B cells from control (Fig. 3A), WP-treated (Fig. 3B) and BSA-treated mice (Fig. 3C) is shown in Fig. 3. Data from six separate experiments (six animals were randomly selected from each group) revealed that the percentage of B cells that migrated specifically toward CCL-21 and CXCL-12 was 19±1.8 and 33±3.1 in control mice and 17±1.6 and 35±3.3 in BSA-treated mice and was significantly increased to 31±2.8 and 49±4.5 in WP-treated mice, respectively (Fig. 3D).

3.5. The administration of WP increases T cells migratory capacity towards CCL-21 and CXCL-12

The percentage of T cells isolated from the spleen and PLNs that specifically migrated was assessed using flow cytometry. For the splenic T cells ($n=6$, six animals were randomly selected from each group), the percentage of cells that showed specific CCL-21- and CXCL-12-mediated migration was 27±2.2 and 42±3.5 in control mice and 29±2.1 and 41±22.9 in BSA-treated mice and was significantly increased to 38±3.4 and 57±4.4 in WP-treated mice, respectively (Fig. 4A). Similarly, for the T cells that were isolated from PLNs ($n=6$), the percentage of cells that showed specific CCL-21- and CXCL-12-mediated migration was 33±2.6 and 38±2.9 in control mice and 35±3.2 and 36±2.9 in BSA-treated mice and was significantly increased to 46±3.7 and 51±4.1 in WP-treated mice, respectively (Fig. 4B).

3.6. BM-DCs from WP-administered mice exhibit increased migratory capacity towards CCL-21 and CXCL-12

Immature DCs were differentiated from the BM of the three groups of mice. DC maturation was achieved using LPS stimulation and confirmed using the detection of CCR7 up-regulation. One representative experiment showed that mature DCs ($CD11c^{+}/CD11b^{+}$) clearly expressed CCR7 (Fig. 5A). In this context, CCR7 expression was greater in WP-treated mice compared to control and BSA-treated mice. The migratory capacity of mature DCs to CCL-21 and CXCL-12 was then measured using a transwell system and analyzed using flow cytometry. Data from five separate experiments revealed that the percentage of DCs that underwent CCL-21- and CXCL-12-mediated specific migration was 42±3.9 and 29±2.7 in control mice and 40±3.6 and 28±2.6 in BSA-treated mice and was significantly increased to 59±3.3 and 44±3.9 in WP-treated mice, respectively (Fig. 5B).

4. Discussion

Although several studies have shown the beneficial effects of WPs on human health, particularly on innate and acquired immunity [18,22], the possible cellular and molecular mechanisms of the action of undenatured WPs remain poorly defined. WPs have utility in many different areas, including effects on bone, muscle, blood, brain, pancreas, immune cells, cancer, infection, metabolism, wound healing, learning and aging [32]. In this study, we monitored changes in blood parameters and leucocyte counts in three groups of mice, control, WP-treated and BSA-treated mice, throughout the experimental period. We observed a significant decrease in the levels of the total lipid profile (cholesterol, triglycerides, HDL and LDL) and free radicals (hydroperoxide and ROS) in the WP-treated group. Similar observations have shown that daily oral administration for 4 weeks of colostrum, which contains a large amount of WPs, significantly decreases total cholesterol and triglyceride levels in both men and women [33]. The mechanisms behind the effects on lipids may be attributed to the effect of WPs on *de novo* cholesterol biogenesis in the liver [34], the inhibition of the expression of genes involved in intestinal fatty acid and cholesterol absorption and synthesis [35], and/or the increase in fecal steroid excretion [36]. Similarly, Micke et al. [17] revealed that undenatured WP supplementation significantly increased the GSH level in several HIV-infected patients. Moreover, milk WP decreases oxygen free radical production in a murine model of chronic iron-overload cardiomyopathy [37]. The current findings indicate a significant increase in the GSH level and

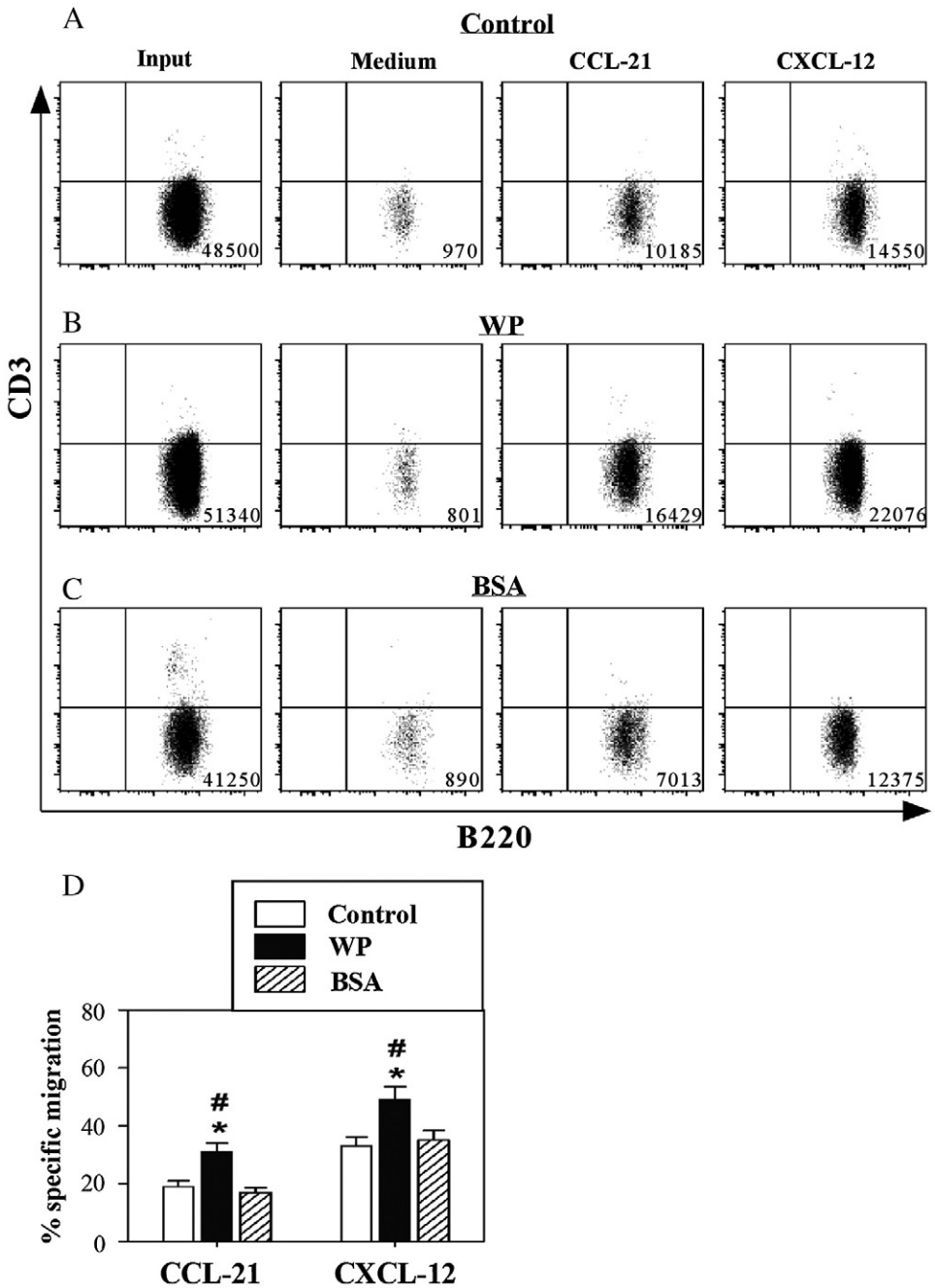

Fig. 3. The impact of WP on splenic B cell chemotaxis. Splenic B cells were isolated from eight mice in each group and analyzed for migration to CCL-21 and CXCL-12. Input populations and migrated cell populations were stained with B220-PE/CD3-FITC. Representative dot plots of input cells and transmigrated cells to medium without chemokine versus medium containing CCL-21 or CXCL-12 for control (A), WP-treated (B) and BSA-treated (C) mice are shown. The numbers of cells in the input and transmigrated B cell population are shown. Data from one representative experiment are shown in panels (A–C). The percentages of B cells from six different mice in control (open bars), WP-treated (black bars) and BSA-treated (hatched bars) groups that specifically migrated to chemokines are shown, and the results are expressed as the mean specific migration±S.E.M.. $^{*}P<.05$, WP vs. control and $^{\#}P<.05$, WP vs. BSA.

WBC count in the WP-treated group. WPC has potential antioxidant activity owing to its ability to increase cellular GSH levels, which is a key step in the antiapoptotic activity of WPC [38].

Cytokines include a large group of glycoproteins that have the capacity to modulate the activity of individual cells under both physiological and pathological conditions [39]. These polypeptides are synthesized in response to microorganisms and other antigens, and they mediate and regulate immune and inflammatory reactions. Several studies have described alterations in the expression of different cytokines after WP supplementation [40,41]. Our data revealed that WP-treated mice showed significant decreases in the levels of circulating IL-1α, IL-1β, IL-10 and TNF-α. There is now a large body of evidence suggesting that the development of diseases is associated with spontaneous increases in proinflammatory cytokines [42]. The ability of WP in decreasing these cytokines enhances immune response, and subsequently, it may be a promising drug candidate for immunomodulation in different diseases. In contrast, significant increases were clearly observed in the levels of IL-2, IL-4, IL-7 and IL-8. Normally, IL-2 and IL-7 act as growth factors for the survival and maintenance of mature functional T cells in the periphery

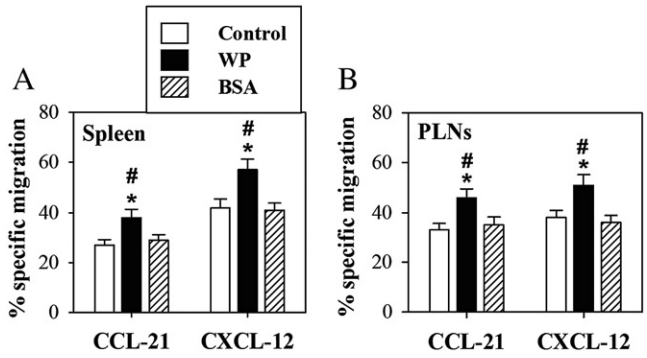

Fig. 4. The impact of WP on T cell chemotaxis. The ability of T cells negatively purified from the spleen (A) or PLNs (B) to migrate in response to CCL-21 and CXCL-12 was measured using transwells and flow cytometry. The percentages of T cells from six different mice in control (open bars), WP-treated (black bars) and BSA-treated (hatched bars) groups that specifically migrated to chemokines are shown, and the results are expressed as the mean specific migration±S.E.M. $^{*}P<.05$, WP vs. control and $^{\#}P<.05$, WP vs. BSA.

[43], whereas IL-4 plays an important role in B cell activation [44]. Another study revealed that lactoferrin could regulate levels of TNF and IL-6, thus decreasing inflammation and, ultimately, mortality [45]. Our results are in agreement with recently published data that revealed that WP stimulated immune cells to release various cytokines, particularly IL-1 [22].

Cell proliferation after Ag activation is an important biological parameter used in the diagnosis of immunodeficiencies in clinical laboratory research and in various fields of lymphocyte research. In this study, the proliferative capacity of lymphocytes following different stimulations was significantly increased in the WP-treated mice. Several studies have focused on the *in vitro* and *in vivo* modulation of lymphocyte proliferation by individual WPs. The *in vitro* proliferation of murine splenic lymphocytes was stimulated by WP, but the effect was reduced following hydrolysis using a trypsin/chymotrypsin mixture [46]. In addition, WP increases osteoblast proliferation, as described by Lee et al. [47]. Lymphocyte recirculation, which is critical for effective immunity, is tightly regulated by the expression of adhesion molecules and chemoattractant receptors on lymphocytes combined with the spatial and temporal expression of ligands for these receptors by a variety of tissue cells [48]. Our results showed that WP increased CCL-21- and CXCL-12-mediated B, T and BM-DC chemotaxis, which is a major finding. Previous studies have shown that CCR7 and CXCR4 are involved in the recruitment of blood-borne leukocytes to sites of inflammation [49]. As receptors, CXCR4 and CCR7 can be considered activation markers [50]. The chemokines CCL-21 and CXCL-12 belong to the family of homeostatic molecules that regulate immune and nonimmune cell homing and survival [51] and the control of lymphoid organogenesis and homeostasis [31]. An *in vitro* study has shown that CXCL-12/CXCR4 engagement is required for the migration of cutaneous DCs [52]. Recently, reports have shown that bovine WP enhances innate immunity by increasing neutrophil chemotaxis [53]. Our data suggest a better efficiency of B, T and DC cell chemotaxis following WP treatment and immune defense against microorganisms and particles. Moreover, the ability of WP to enhance the release of GSH and cytokines, down-regulate the lipid profile and increase immune cell chemotaxis and proliferation could be used to accelerate the destruction of foreign pathogens and establish an effective immune response. In conclusion, the present data expand our knowledge about the role of WP supplementation in enhancing the immune response and the mechanisms that underlie this response, suggesting that WP may be a promising drug candidate for immunomodulation.

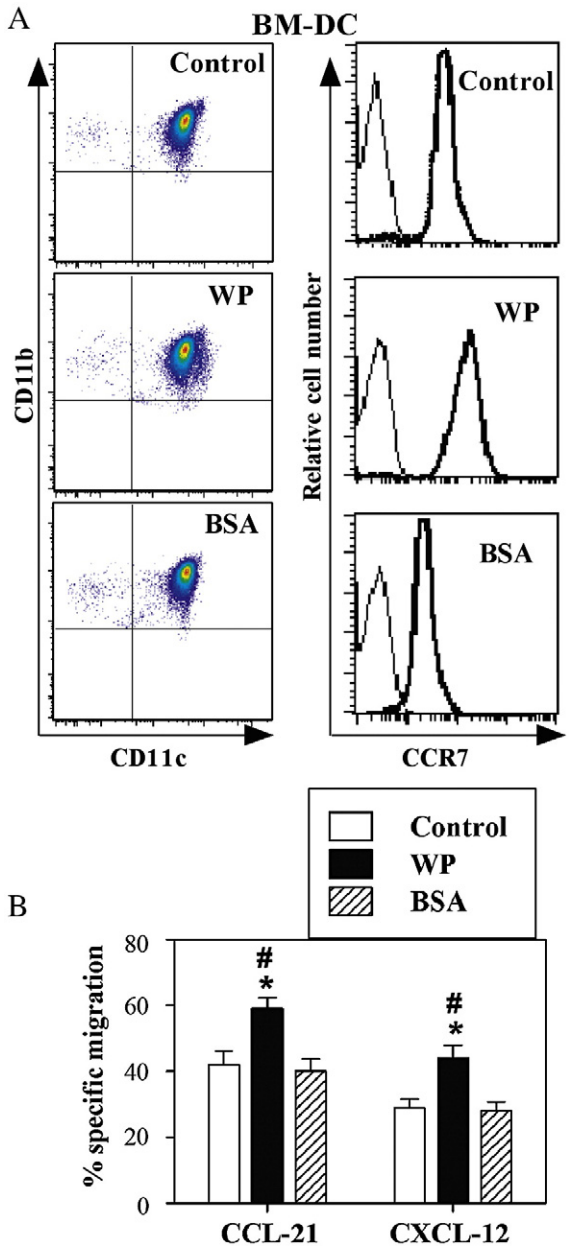

Fig. 5. The impact of WP on the chemotaxis of BM-DCs. DCs were generated from bone marrow and were CD11c/CD11b double-positive. One representative experiment out of five is shown (A) to illustrate the maturation of DCs (up-regulation of CCR7 expression) after stimulation with LPS. The transmigration of mature DCs to CCL-21 and CXCL-12 was measured using a transwell system using flow cytometry. The percentages of DCs from five different mice in control (open bars), WP-treated (black bars) and BSA-treated (hatched bars) groups that specifically migrated to chemokines are shown, and the results are expressed as the mean specific migration±S.E.M. $^{*}P<.05$, WP vs. control and $^{\#}P<.05$, WP vs. BSA.

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