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Tocmo, R. ORCID: https://orcid.org/0009-0007-4850-977X, Balagiannis, D., Quinn, E., Finnegan, D., Collins, M. and Loscher, C. E. (2024) Protein hydrolysates from quinoa (Chenopodium quinoa Willd.) modulate macrophage polarization and the expression of surface antigen molecules. Sustainable Food Proteins, 2 (4). pp. 282-296. ISSN 2771-9693 doi: 10.1002/sfp2.1042 Available at https://centaur.reading.ac.uk/119195/

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To link to this article DOI: http://dx.doi.org/10.1002/sfp2.1042

Publisher: Wiley

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Received: 7 December 2023 | Revised: 7 October 2024 | Accepted: 8 October 2024

DOI: 10.1002/sfp2.1042

ORIGINAL ARTICLE

AOCS WILEY

Protein hydrolysates from quinoa (*Chenopodium quinoa* Willd.) modulate macrophage polarization and the expression of surface antigen molecules

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Funding information
European Union's Horizon 2020 Research and Innovation Programme under the Marie Skodowska-Curie Actions Grant Agreement, Grant/Award Number: MF20210255; Deparment of Food Science and Nutrition, University of Reading; Enterprise Ireland Grant Agreement, Grant/Award Number: TC20180025

Abstract

Quinoa (*Chenopodium quinoa* Willd.) is considered an exceptional source of high-quality protein and may be a good precursor of bioactive components with immunomodulatory effects. Quinoa protein-enriched fraction (QPF) was isolated from quinoa seeds via alkaline extraction and isoelectric point precipitation and hydrolyzed at the optimum temperature and pH of two food-grade proteases to produce QPF hydrolysates (QPFH). SDS-PAGE and LC–MS analyses showed that low-molecular weight peptides were present in hydrolyzed proteins and that the enzymes effectively hydrolyzed high-molecular weight proteins evidenced by significantly higher amino acid levels in QPFH. J774A.1 macrophages and mouse bone-marrow-derived macrophages (BMDMs) were polarized into an M1-like (pro-inflammatory) or M2-like (anti-inflammatory) state in the absence or presence of QPF or QPFH. Our results showed that QPFH attenuated M1-like response as demonstrated by a significantly lowered secretion of pro-inflammatory cytokines (IL-6, TNF-α, IL-12p40, and IL-27p28) and nitric oxide (NO) levels. Co-treatment with QPFH significantly boosted IL-10 and arginase activity in both M1-like and M2-like cells indicating that the samples may promote a phenotypic switch to M2-like macrophages. Furthermore, QPFH inhibited pro-inflammatory cytokines in Loxoribine (LOX)-activated bone marrow-derived dendritic cells (BMDCs) and inhibited the same cytokines during a 7-day DC maturation experiment. QPF but not the QPFH influenced the expression of surface antigen molecules in macrophages by decreasing the frequency of MHCI and CD86 expressing cells. Taken together, these findings reveal novel mechanisms that demonstrate the potential of quinoa protein hydrolysates in the development of immunomodulatory functional foods.

KEYWORDS
immunomodulation, M2-like macrophages, macrophage polarization, quinoa protein hydrolysates, surface antigen molecules

Abbreviations: 7-AAD, 7- aminoactinomycin D; BMDC, bone marrow-derived dendritic cells; BMDM, bone marrow-derived macrophages; BSA, bovine serum albumin; CD, cluster of differentiation; ESI, electrospray ionization; FACS, fluorescence-activated cell sorting; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; GM-CSF, granulocyte-macrophage colony-stimulating factor; HILIC, hydrophilic interaction chromatography; IFN-y, interferon-gamma; IL, interleukin; LOX, loxoribine; LPS, lipopolysaccharide; M1/M2, macrophage-like 1/2; MCP-1, monocyte chemoattractant protein; MFI, mean fluorescence intensity; MHCI/II, major histocompatibility complex; MIP-1α/2, macrophage inflammatory protein; MRM, multiple reaction monitoring; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; PBS, phosphate buffered saline; PE, phycoerythrin; PTFE, polytetrafluoroethylene; QPC-FL, QPF hydrolyzed by Flavourzyme™; QPF, quinoa protein-enriched fraction; QPF-PN, QPF hydrolyzed by papain; rMCSF, recombinant macrophage colony-stimulating factor; RPMI, Roswell Park Memorial Institute; TLR, toll-like receptor; TNF-α, tumor necrosis factor-alpha.

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INTRODUCTION

Macrophages and dendritic cells (DCs) do not only play a role in innate immunity, but they are also responsible in maintaining homeostasis in the intestinal mucosa and in the development of intestinal inflammation. In the context of chronic inflammation, understanding how the distinct macrophage effectors M1/M2 (the two major and opposing activities of macrophages) can be modulated could potentially delineate the next steps in supporting the treatment of intestinal inflammatory diseases (Han et al., 2021). M1-like macrophages inhibit cell proliferation and promote tissue damage while the M2-like macrophages promote tissue repair and cell proliferation (Mills, 2012). Remarkably, the molecules primarily responsible for these opposing activities—"fight" (nitric oxide) or "fix" (ornithine)—both arise from arginine, and via enzymatic pathways (iNOS and arginase) that downregulate each other. Furthermore, M1-like and M2-like macrophages promote T helper (Th) 1 and Th2 responses, respectively (Wang et al., 2014). Thus, the effector activities of M1/M2 demonstrate the importance of innate immunity and how it is linked to adaptive immunity (Mills, 2012).

Pre-clinical evidence has started to define the beneficial effects of protein hydrolysates in modulating immune responses (Kiewiet et al., 2018) and in reducing the allergenicity of dietary proteins (Calcinai et al., 2022; Liang et al., 2021). Therefore, it is valuable to assess the immune-modulating abilities of other sources of protein hydrolysates by measuring at least one or a combination of parameters of either innate or adaptive immunity (Kaminogawa & Nanno, 1900). Studies have revealed that quinoa protein hydrolysate (QPH) may possess enhanced levels of free amino acids and bioactive peptides. For example, the study by Nongonierma et al. (2015) reported that QPH prepared with two enzyme preparations (papain and a microbial papain-like enzyme) had a significantly higher concentration of free amino groups than the corresponding protein isolate. Consequently, studies have reported diverse biological activities of QPH prepared using various enzymes such as alcalase, papain, and bromelain (Mir et al., 2021; Mudgil et al., 2019; Nongonierma et al., 2015; Shi et al., 2019).

Building on the existing evidence on the bioactive potential of quinoa protein, the present study focuses on the potential of QPH in modulating innate immune responses in vitro. Specifically, this study investigated the impact of the water-soluble components of QPF hydrolysates (QPFH) on macrophage polarization and DC activation. We provide in vitro evidence that QPFH may possess enhanced immunomodulatory effects compared to the unhydrolyzed protein. Specifically, we show that QPFH could drive macrophage polarization into the anti-inflammatory M2-like phenotype, inhibit pro-inflammatory cytokine production, enhance IL-10 levels, and modulate expression of surface antigen molecules in macrophages and DCs.

EXPERIMENTAL PROCEDURES

Chemicals and reagents

Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS) and Penicillin/Streptomycin were purchased from Invitrogen. RPMI-1640 was purchased from Gibco. Lipopolysaccharide (LPS) isolated from *Escherichia coli*, serotype R515, was purchased from Enzo Life Sciences. Loxoribine (LOX) was purchased from Invitrogen. CellTiter 96® Aqueous One Solution was purchased from Thermo Fisher Scientific. DuoSet cytokine and chemokine ELISA kits were purchased from R&D Systems. Proteases (Flavourzyme® from *Aspergillus oryzae* [≥500 U/g], Novozyme Corp) and Papain from papaya latex were purchased from Sigma-Aldrich.

Flour preparation and isolation of protein-enriched fraction

Quinoa seeds were provided by Tirlán (formerly Glanbia Ireland). The seeds were cleaned of impurities and foreign materials and stored in a dry place at room temperature (22 ± 2°C) for further analysis. Whole seeds (500 g) were washed with deionized water 3–5 times or until there was no foam to remove saponins, then freeze-dried 24 h. Freeze drying was preferred over oven drying to keep the seeds from potential contamination with prolonged air exposure. The whole seeds were ground into flour using a coffee grinder with a 60-mesh screen. Extraction of quinoa protein-enriched fraction (QPF) was carried out according to a previous study (Nongonierma et al., 2015; Scanlin et al., 2009) with some modifications. Briefly, quinoa flour was suspended in water (10% w/v) and adjusted to pH 9.0 with 1 M NaOH. The mixture was heated at 40°C in a water bath with continuous agitation for 2 h and centrifuged at 2000×*g* for 20 min. The supernatant was collected and adjusted to pH 4.5 with 0.1 M HCl and centrifuged for 20 min at 2000×*g*. The precipitated protein-enriched fraction was resuspended in water, adjusted to pH 7.4, freeze-dried (6L FreeZone Freeze Dryer 77520-01, Labconco, UK) and stored at −20°C until use. The moisture content was measured using the AACCI approved Method 44-19.01. The protein content of the water-soluble fractions was measured using Better Bradford Protein Assay Kit (NBS Biologicals) following the manufacturer's instructions.

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Preparation of QPFH

To obtain hydrolysates, freeze-dried QPF was treated for 3 h with 2% (w/w) of Flavourzyme™ (FL), a protease from *Aspergillus oryzae* or Papain (PN) to produce two hydrolysates, QPC-FL, QPC-PN, and an unhydrolyzed control QPF. The incubation was carried out at optimum pH and temperature for each protease: FL (pH 7.0, 50°C) and PN (pH 7.0, 60°C). Control QPF dispersions were prepared under the same incubation conditions and inactivation treatment, but without enzyme addition. At the end of the hydrolysis, proteases were inactivated by heating the protein suspension in a hot water bath at 90°C for 10 min. The hydrolyzed suspensions (hydrolysates) were freeze-dried and stored at −20°C. To prepare samples for the cell culture experiments, freeze-dried QPF or QPFH (pH 7.4) was resuspended in HPLC-grade water (10% w/v) in 50-ml centrifuge tubes and put in a shaker-incubator at a shaking speed of 200 rpm. The samples were centrifuged at 4000×*g* for 20 min and the supernatant (water-soluble fraction) was transferred into new centrifuge tubes and freeze-dried.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

Proteins in the QPF water extracts (QPF-WE) or QPFH water extracts (QPFH-WE) were resolved under reducing conditions on a Mini-PROTEAN Tetra Cell system (Bio-Rad Laboratories) according to the manufacturer's instructions using a 12% polyacrylamide gel. The protein solution (supernatant of the centrifugation/decanting step, 8 g protein equivalents L^{-1}) was diluted with a 2× Laemmli sample buffer (1:1 v/v). Proteins were visualized by staining with Coomassie brilliant blue (0.025% w/v in 10% acetic acid) and destained in 40% methanol and 10% acetic acid solution. Precision Plus Protein Dual Color Standards (Bio-Rad) was used as molecular weight standards.

Tricine-SDS-PAGE

Tricine-SDS-PAGE was carried out to visualize peptides present in 10 and 3 kDa fractions obtained by ultrafiltration. Peptide-rich fractions from QPF-WE or QPFH-WE were isolated using Amicon® Ultra Centrifugal Filter (Sigma-Aldrich) and freeze-dried. Freeze dried samples (8 g protein equivalents L^{-1}) were dissolved in Tricine sample buffer (16107391, Bio-Rad) and boiled (100°C) for 5 min. Samples (20 μL) were loaded into a 16.5% Criterion™ Tris-Tricine polyacrylamide gel and electrophoresed in Mini-PROTEAN Tetra Cell system (Bio-Rad) at a constant voltage of 170 mV. After electrophoresis, the gel was stained with 0.025% (w/v) Coomassie Blue G-250 in 10% (v/v) acetic acid for 2 h, and destained in 40% methanol and 10% acetic acid solution.

Generation of bone marrow-derived macrophages (BMDMs) and dendritic cells (BMDCs)

Bone marrow was isolated from 6 to 12 weeks old adult female BALB/c mice. Mice were euthanized in a CO_2 chamber followed by cervical dislocation. Femurs and tibias were isolated in sterile conditions, and the bone marrow was flushed into a sterile 10-cm petri dish using RPMI growth media supplemented with 10% heat-inactivated FBS and 2% Penicillin/Streptomycin (100 U/mL) (Sigma). A single cell suspension was prepared by passing the bone marrow suspension through a 19-gauge needle followed by centrifugation (5 min, 250×*g*). The bone marrow pellet was then resuspended in complete RPMI media (25 ng/mL rM-CSF, 10% heat-inactivated FBS, 10% Penicillin/Streptomycin) and plated onto cell culture petri dish at a ratio of one bone per plate (10 mL media/plate). Cells were incubated for a total of 6 days at 37°C and 5% CO_2 levels, adding 10 mL of complete RPMI media on Day 3. To generate BMDCs, bone marrow cells were suspended in complete RPMI media (20 ng/mL GM-CSF, 10% heat-inactivated FBS, 10% Penicillin/Streptomycin) for 7 days, adding 10 mL of complete media on Day 4. On Day 6 (BMDMs) or Day 7 (BMDCs), single-cell suspensions were stained with fluorochrome-conjugated specific antibodies (Table S1) and Flow cytometric analysis (FACSAria flow cytometer, BD Biosciences) of cell surface markers was carried out on 7-AAD-negative cells (to exclude dead cells) to assess cell differentiation and purity (Figures S3 and S4).

Cell culture and viability assay

The murine macrophage cell line J774A.1 was obtained from the European Collection of Cell Cultures (ECACC; Salisbury, UK). J774 macrophages were cultured in Dulbecco's modified Eagle's medium (DMEM) with glutamine (Sigma-Aldrich) supplemented with 10% heat-inactivated FBS (Invitrogen™), 100 U/mL penicillin, and 100 μg/mL streptomycin, (Gibco, Thermo Fisher Scientific, Ireland). The murine DC line JAWS II (CRL-11904, ATCC) is an immature DC line derived from a p53 growth suppressor gene deficient C57BL/6 mice, which have been reported to be induced to become an activated DC line. JAWSII cells were cultured in α-MEM (Invitrogen™) supplemented with 20% non-heat inactivated FBS, 100 μg/mL/100 μg/mL Penicillin/Streptomycin, 50 μg/mL Gentamicin, and 50 μg/mL

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murine recombinant GM-CSF (Sigma-Aldrich). All cell culturing procedure were carried out using aseptic techniques in a class II laminar airflow unit (MSC-Advantage™ Class II, ThermoFisher Scientific, Dublin, Ireland). Cells were maintained in a 37°C incubator with 5% CO_2 and 95% humidified air (Forma™ Steri-cycle CO_2 incubator, ThermoFisher Scientific, Dublin, Ireland).

Cell viability was evaluated using a standard MTS assay. In addition, the effect of the samples (2 mg/mL) on the cell viability of BMDMs was assessed by flow cytometry using the fluorescent DNA intercalator 7-AAD. Cells exposed to samples for 22 h were washed with FACS buffer and stained with 7-AAD (BD Pharmingen) for 15 min. Cell events were acquired on a FACSAria flow cytometer and FlowJo software was used to analyze FCS files.

M1-like and M2-like macrophage polarization

J774 macrophages or BMDMs were seeded in 24-well plates at 1×10^6 cells/well (200 μL/well). After 1 h, cells were exposed to 2 mg/mL QPF-WE or QPFH-WE and incubated for 2 h at 37°C. Cells were then further treated with either M1-like polarizing or M2-like polarizing agents or left untreated (M0). To induce M1-like polarization, cells were exposed to LPS (100 ng/mL) and 20 ng/mL IFN-γ. To polarize M0 to the M2-like phenotype, cells were treated with IL-4 (20 ng/mL) and IL-13 (20 ng/mL) (Orecchioni et al., 2019). Plates were then incubated at 37°C and 5% CO_2 levels for 22 h. After 22 h, cell culture supernatants were collected for the analysis of cytokine, chemokines, or nitric oxide (NO). To evaluate the effect of QPF-WE or QPFH-WE on cell surface antigen molecules, 1.5×10^6 cells/well were seeded in 6-well plates and the same pretreatment and polarization procedure as described was carried out. Single-cell suspensions were then stained with fluorochrome-conjugated specific antibodies (Table S1) for and analyzed by Flow cytometry.

Cytokine and chemokine measurements

Cell culture supernatants were analyzed for cytokines (IL-6, TNF-α, IL-10, IL-12p40, IL-27p28, and IL-23) and chemokines (MIP-1α, MIP-2, and MCP-1) by enzyme-linked immunosorbent assay (ELISA) following the manufacturer's instructions. When necessary, samples were diluted with 1% BSA/PBS-Tween 20. The absorbance was measured in a microplate reader (VERSAmax Microplate reader, Molecular Device, Berkshire, UK) at 450 nm. Concentrations were calculated against corresponding standard curves.

NO measurement

The effect of samples on NO production was analyzed by measuring nitrite (NO_2^-), a stable metabolite of NO, using the Griess assay following the manufacturer's instruction. Assessment of NO production was recorded as relative colorimetric changes measured at 540 nm and NO_2-levels were calculated against a standard curve.

Arginase activity assay

Arginase activity was determined in J774A.1 cells and BMDMs using the Arginase Activity Assay Kit (Sigma, MAK112) following manufacturer's protocols. Briefly, cells were lysed in a low-stringency lysis buffer (provided in the kit). Cell lysates (10–30 μL) were transferred into a 96-well plate, followed by the addition of an arginine substrate buffer (provided in kit) and incubation at 37°C for 2 h. The reaction was stopped by the addition of Urea reagent. The plate was incubated for a further 1 h at room temperature, followed by determination of absorbance at 430 nm. Arginase activity in units/L was calculated as indicated by the manufacturer based on the absorbance of a 1 mM urea standard.

Flow cytometric analysis of surface markers

Following the M1/M2 polarization experiments, staining of single-cell suspension was performed at 4°C for 20 min after Fc blocking (TruStain FcX™ PLUS, anti-mouse CD16/32 clone S17011E, Biolegend) for 15 min with the following monoclonal antibodies: FITC-conjugated rat anti-mouse MHCII (clone: M5/114.15.2; BD), PE-conjugated anti-MHCI (clone: AF6-88.5.5.3, eBioscience), PE-conjugated Hamster anti-mouse CD80 (clone: 16-10A1, BD), and FITC-conjugated Rat anti-CD86 (clone: GL1, BD) for 30 min. The 7-AAD (Cat. no. 00-6993, eBioscience, 0.25 μg) was used for gating out dead cells. Fluorescence levels were measured on the 7-AAD-negative population by flow cytometry (FACSAria, BD Biosciences) analyzing 10,000 events per sample. A flow gating strategy is provided Figures S3 and S4. In separate experiments, cell surface markers in JAWSII cells were stained using the same conjugated antibodies and analyzed by flow cytometry. All data were analyzed with FlowJo software (BD Bioscience, version 10).

Free amino acid analysis by LC–MS

QPF-WE or QPFH-WE (10 mg) were added in a 15 mL falcon tube containing a ceramic bead and dissolved in

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10 mL of aqueous solution of nor-valine 10 μM (internal Standard). The mixture was rigorously stirred and homogenized (FastPrep-24 5G, MP Biomedicals, LLC, UK) for three cycles of 2 min each, with a 2 min intermission between each mixing cycle. Then, it was centrifuged at 10,000×*g* and 4°C for 10 min, and the supernatant was filtered through a 0.2 μm PTFE syringe filter (Fischer Scientific, UK) into an LC–MS vial. Instrumental analysis of the free amino acids was performed in an Agilent 1200 HPLC system coupled to a 6410 triple quadrupole mass spectrometer. For quantification, calibration curves were built by running standard solutions (made from a stock solution of AAS18, Sigma Aldrich, UK) containing mixtures of 10 μM of nor-valine with the following amino acids on a range of 0–25 μM: Alanine (Ala), Arginine (Arg), Aspartic Acid (Asp), Glutamic acid (Glu), Glycine (Gly), Histidine (His), Isoleucine (Ile), Leucine (Leu), Lysine (Lys), Methionine (Met), Phenylalanine (Phe), Proline (Pro), Serine (Ser), Threonine (Thr), Tyrosine (Tyr), Valine (Val). Chromatographic separation was achieved with a Synchronis HILIC precolumn (10 × 4.6 mm i.d., 3 μm) coupled to a Synchronis HILIC column (150 × 4.6 mm i.d., 3 μm; ThermoFisher Scientific, Waltham, MA, USA). The column temperature was set at 20°C while injection volume was 5 μL. An eluent gradient program was employed to optimize separation: Eluent [A] was

TABLE 1 Total protein content of quinoa protein-enriched fraction and its protein hydrolysates.

	QPF	QPF-FL	QPF-PN
Moisture content (%)	3.34.3 ± 0.62	4.76 ± 2.32	3.82 ± 1.90
Protein content (%)	42.8 ± 4.93	47.48 ± 6.88	45.9 ± 4.55

Note: Values are means ± SD ($n = 3$).

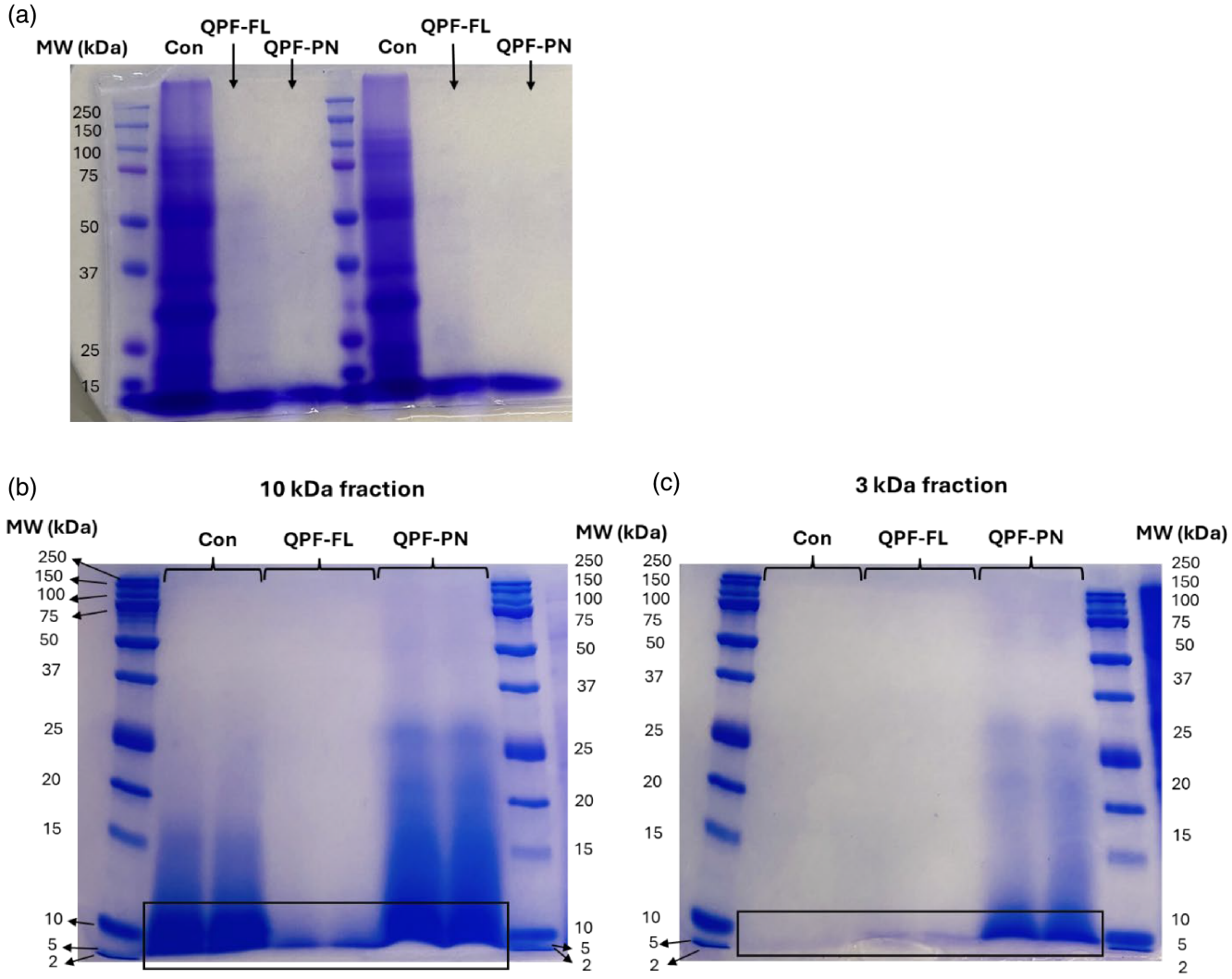

FIGURE 1 Protein and peptide profiles of the water-soluble extracts from quinoa protein-enriched fraction (QPF) and the hydrolyzed proteins, QPF-FL and QPF-PN. (a) Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) profiles of the water-soluble extracts from the three samples; (b) Tris-Tricine SDS-PAGE of 10 and 3 kDa fraction obtained by ultrafiltration using the using Amicon® Ultra Centrifugal Filters.

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composed of 5 mM ammonium formate with 0.5% formic acid in water; Eluent [B] contained 5 mM ammonium formate with 0.5% formic acid in acetonitrile: water (9:1, v: v). Total run time was 13 min: from 0 to 8 min [B] increased from 10% to 40%, from 8 to 9 min [B] decreased from 40% to 10%, and from 9 to 13 min [B] was kept constant at 10%. MS/MS detection was achieved with ESI at positive polarity mode and the following source settings: gas temperature 330°C, gas flow 13 L/min, nebulizer pressure 40 psi and capillary voltage 4000 V. The MRM transitions that were employed for the detection and quantification of the amino acids were as previously reported (Sood et al., 2024).

Statistical analysis

Statistical analyses were performed by one-way ANOVA and the Tukey's post hoc test using the GraphPad Prism software (version 10.0.1, GraphPad Software, San Diego, CA, USA). Unless otherwise specified, results are illustrated as mean values ± SD. P values less than 0.05 were considered statistically significant and indicated by asterisks: *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$; ****$p \leq 0.0001$. Statistical analysis of the amino acid profiles was carried out XLSTAT (Lumivero, USA). Multiple pairwise comparisons were conducted with Excel, using *t*-test ($p \leq 0.05$).

RESULTS AND DISCUSSION

The use of protein hydrolysates with immunomodulatory effects is an attractive opportunity to manage immune-related diseases though protein nutrition in functional foods. We investigated the immunomodulatory effects of QPF-WE and QPFH-WE on macrophage polarization and DC activation.

Protein and amino acid profile

The total protein contents of the protein fraction obtained were in the range of 42%–47% (Table 1), which is below the 50% protein contents of typical food protein concentrates. These values are relatively lower than previous values reported, for example, 52.40% and 65.01% (Vilcacundo et al., 2017). This may be due to the fact that the extraction was carried out using a coffee grinder and the flour obtained may have larger particle size and lower volume: surface ratio, leading to a lower protein purity after extraction. Therefore, we refer to the protein-enriched fractions we obtained as QPF or QPFH. From here on, the abbreviations QPF-FL and QPF-PN refer to the water-soluble extracts of enzyme hydrolyzed QPF (QPF-FL—hydrolyzed by Flavourzyme™; QPF-PN—hydrolyzed by Papain). The protein values we obtained were are consistent with

TABLE 2 Free amino acid profiles of the water-soluble extracts of quinoa protein fraction (QPF) and hydrolyzed QPF, QPF-FL and QPF-PN analyzed by LC–MS.

Amino acids	QPF (mg/100 g)	QPF-FL (mg/100 g)	QPF-PN (mg/100 g)	Significance of sample region (*p*-value)	
Alanine (Ala)	41.3 ± 4.65^{c}	10.76 ± 2.21^{a}	72.8 ± 7.90^{b}	<0.001	***
Arginine (Arg)	508 ± 50.2^{c}	519.19 ± 19.89^{c}	1039 ± 151.4^{b}	<0.001	***
Asparagine (Asn)	20.3 ± 4.28^{c}	112.5 ± 4.75^{a}	73.1 ± 5.34^{b}	<0.001	***
Aspartic Acid (Asp)	56.8 ± 12.1^{c}	49.25 ± 3.55^{c}	118 ± 13.4^{b}	<0.001	***
Glutamic acid (Glu)	104 ± 3.51^{c}	22.25 ± 3.00^{a}	146 ± 15.4^{b}	<0.001	***
Glutamine (Gln)	37.7 ± 2.07^{c}	86.7 ± 3.73a	52.4 ± 3.26^{b}	<0.001	***
Glycine (Gly)	27.0 ± 12.2^{c}	103.09 ± 9.03^{b}	151 ± 37.2^{b}	<0.001	***
Histidine (His)	47.4 ± 4.66^{c}	24.45 ± 1.42^{c}	59.3 ± 5.4^{b}	<0.001	***
Isoleucine (Ile)	25.9 ± 1.73^{c}	11.49 ± 0.64^{b}	33.0 ± 2.3^{b}	<0.001	***
Leucine (Leu)	61.5 ± 2.54^{c}	191.85 ± 14.79^{a}	294 ± 18.1^{b}	<0.001	***
Lysine (Lys)	37.6 ± 3.15^{c}	108.79 ± 6.29^{a}	224 ± 17.6^{b}	<0.001	***
Methionine (Met)	20.6 ± 2.28^{c}	37.97 ± 1.96^{c}	57.4 ± 5.9^{b}	<0.001	***
Phenylalanine (Phe)	96.7 ± 14.9^{c}	78.05 ± 8.64^{c}	231 ± 22.0^{b}	<0.001	***
Proline (Pro)	19.0 ± 1.74^{b}	7.92 ± 0.44^{a}	17.6 ± 1.4^{b}	<0.001	***
Serine (Ser)	46.8 ± 3.17^{c}	60.48 ± 2.33^{a}	110 ± 15.2^{b}	<0.001	***
Threonine (Thr)	11.1 ± 1.03^{c}	13.25 ± 1.26^{c}	26.5 ± 3.93^{b}	<0.001	***
Tryptophan	18.1 ± 1.12^{c}	35.14 ± 4.02^{a}	38.3 ± 3.25^{a}	<0.001	***
Tyrosine (Tyr)	76.5 ± 11.4^{c}	46.36 ± 4.40^{c}	111. ± 6.36^{b}	<0.001	***
Valine (Val)	32.9 ± 2.73^{c}	23.38 ± 2.48^{c}	44.5 ± 3.45^{b}	<0.001	***

Note: Signification codes: 0 < *** < 0.001 < ** < 0.01 < * < 0.05 < ns <1. Values are means ± SD ($n = 3$). The values with a different superscript (a–c) within a row are significantly different by paired t-test ($p < 0.05$).

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those reported by Nongonierma et al. (2015). Hydrolysis of protein in QPF was observed on the SDS-PAGE profiles (Figure 1a). The bands corresponding to the intact proteins in QPF at MW 15–250 kDa disappeared in QPF-PN, while very faint bands can be observed in QPF-FL. This indicates that FL and PN were efficient in hydrolyzing these higher-molecular weight proteins at the hydrolysis conditions used. Tricine-SDS-PAGE of 10 kDa fractions obtained by ultrafiltration revealed a thick band, which was not present in the QPF-FL, but present in QPF-PN.

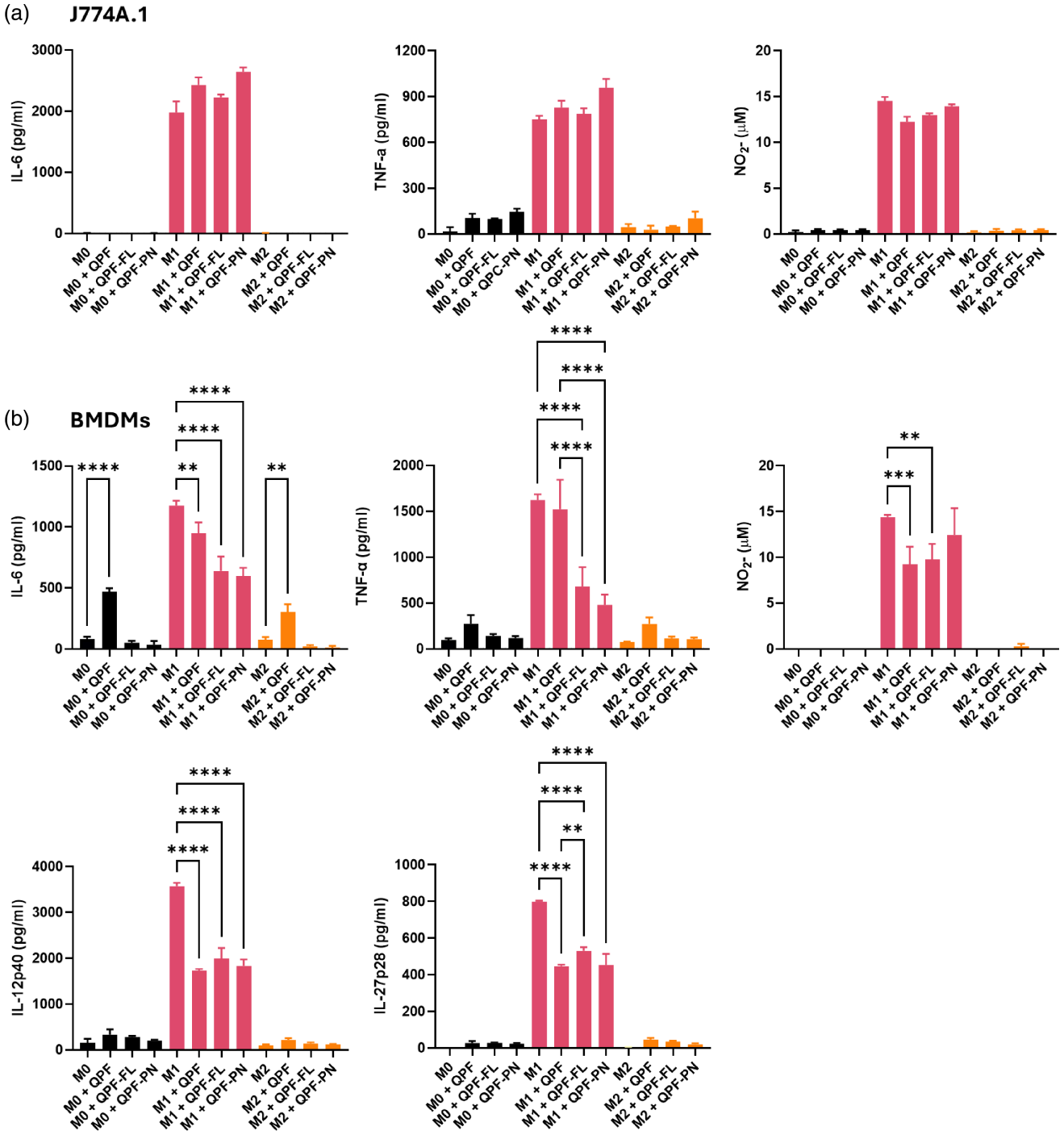

FIGURE 2 Impact of QPF and its enzyme hydrolysates on cytokine production. Cytokine production in J774A.1 (a) and BMDMs (b) cells upon polarization into M0 (naïve), M1-like, or M2-like phenotypes in the presence or absence (control) of water extracts from QPF, QPF-FL, or QPF-PN. Data are expressed as mean values ± SD (one-way ANOVA with Tukey's post-test) from triplicate experiments. Asterisks indicate statistically significant differences (****$p < 0.0001$; ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$) of treatments with the extracts compared to corresponding untreated controls. BMDM, bone marrow-derived macrophages; QPF, quinoa protein-enriched fraction; QPF-FL, QPF hydrolyzed by Flavourzyme; QPF-PN, QPF hydrolyzed by Papain.

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This suggest that FL was able to hydrolyze the protein at 10 kDa and that PN, although it appears less thicker than the control, does not seem to have any significant hydrolyzing effect on this protein. It can be noted that there is a band just below 10 kDa for FL in the 10 kDa gel suggesting presence of lower-molecular weight peptides. It can also be observed that the 10 kDa fraction from QPF-PN has bands at molecular weights over 10 kDa, suggesting that fractionation using 10 kDa ultrafilters may not be as efficient as expected to exclude all higher-molecular weight proteins. Therefore caution is needed in interpreting the results shown in the 10 kDa gel. On the other hand, the 3 kDa gel shows no proteins below 5 kDa for QPF. A thin band between 2 and 5 kDa can be observed for FL, while a much thicker band can be seen between 2 and 5 kDa for the PN. These results suggest that the main differentiating component between the unhydrolyzed and hydrolyzed samples were the protein bands that appear to have a molecular weight between 2 and 5 kDa. These results further suggest that these 2-5 kDa peptides maybe the differentiating components that contribute to the immunomodulatory effects of QPF-FL and QPF-PN. The concentrations of the amino acids of QPF-PN (Table 2) were mostly significantly higher than those of the control. Similarly, some of the amino acids in QPF-FL were higher than those of the control. This demonstrates an enzyme-dependent effect clearly related to the differences in their hydrolytic activity (Figure 2).

QPF-FL and QPF-PN modulate cytokine and chemokine production in M1/M2 J774 cells and BMDMs

We found that QPF-FL and QPF-PN (at a nontoxic dose, Figures S1 and S2) had strong inhibitory effects on inflammatory cytokines (IL-6, TNF-α, IL-12p40, and IL-27p28) and NO in M1-like polarized BMDMs (Figure 1). It is notable that both QPF-FL and QPF-PN had significantly more potent inhibitory effects than the unhydrolyzed QPF. In addition, the hydrolysates downregulated two chemokines, MIP-1α and MCP-1, in M1-like macrophages (Figure 3). These cytokines are recognized signature molecules that are induced when macrophages undergo a classical M1-like switching (Orecchioni et al., 2019). M1 macrophages play an essential role in the development of intestinal inflammation. For example, in the colon of patients with ulcerative colitis (UC), M1 macrophages play a dominant role causing an excessive accumulation of pro-inflammatory factors, leading to the damage of the intestinal epithelial barrier and the imbalance of immune homeostasis (Grimm et al., 1995; Zhang et al., 2023). Our results, therefore, suggest the potential of the QPF-FL and QPF-PN to dampen the activity of M1-like macrophages and may be anti-inflammatory in the context of existing intestinal inflammation. However, it is notable that the levels of IL-10 was lower in BMDMs (but not in J774 cells) and DCs treated with the hydrolyzed samples (QPF-FL/PN) compared to the IL-10 levels of cells exposed to QPF (unhydrolyzed), although it can be seen that hydrolyzed samples were still able to drive IL-10 expression significantly higher versus the M1 or M2 controls. This suggests that enzymatic hydrolysis may contribute to lowering the anti-inflammatory effect of QPF through IL-10 inhibition. This observation needs to be validated in in vivo experiments as IL-10 is a potent anti-inflammatory cytokine that prevents inflammatory and autoimmune pathologies.

The anti-inflammatory peptide lunasin and the proteins chenopodin and albumin in quinoa, are known to possess anti-inflammatory properties (Capraro et al., 2020;

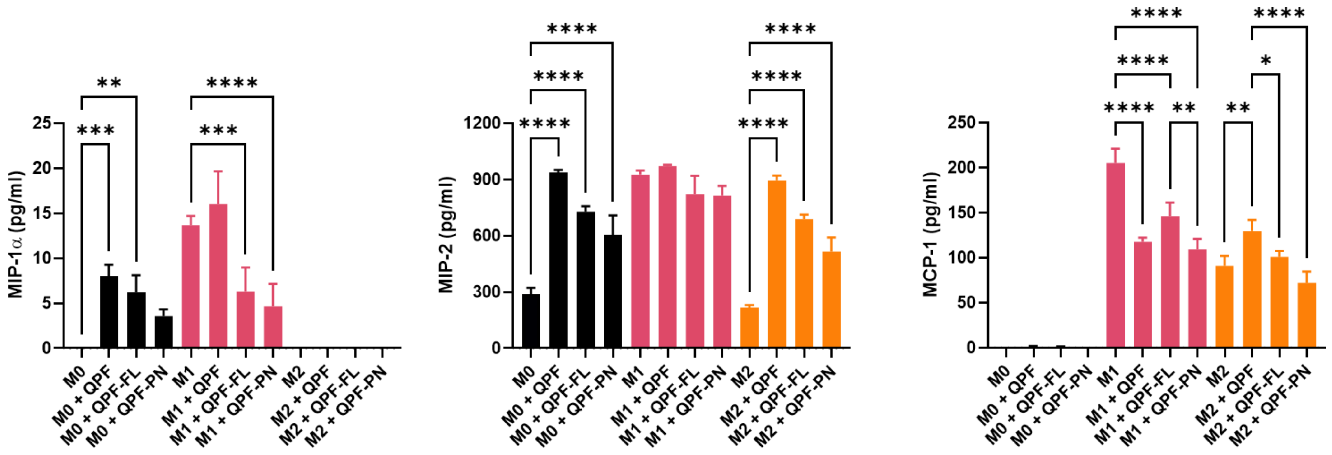

FIGURE 3 Impact of QPF and its hydrolysates on chemokine production. Chemokine production in BMDMs upon polarization into M0 (unpolarized), M1-like, or M2-like phenotypes in the presence or absence (control) of water extracts from QPF, QPF-FL, or QPF-PN. Data are expressed as mean values ± SD (one-way ANOVA with Tukey's post-test) from triplicate experiments. Asterisks indicate statistically significant differences (****$p < 0.0001$; ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$) of treatments with the extracts compared to corresponding untreated controls. BMDM, bone marrow-derived macrophages; QPF, quinoa protein-enriched fraction; QPF-FL, QPF hydrolyzed by Flavourzyme; QPF-PN, QPF hydrolyzed by Papain.

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Pompeu et al., 2022; Ren et al., 2017). In a previous study, quinoa protein and its hydrolysates generated using proteases (PN, pepsin, and pancreatin) were shown to possess high anti-inflammatory activities in RAW264.7 macrophages (Shi et al., 2019). In vivo studies have also demonstrated the anti-inflammatory effect of quinoa (Pompeu et al., 2022; Wang et al., 2023; Zhong et al., 2023). These studies prompted us to investigate whether QPF or QPF-FL/PN can modulate a switch in macrophage polarization from the pro-inflammatory M1-like type to the anti-inflammatory M2-like state. Results from the Tricine-SDS-PAGE of 3 kDa fractions revealed band at 2–5 kDa that were distinct from that of the control (Figure 1b,c). These bands may represent peptides that could potentially be the major components responsible for the observed M2-like polarizing effects of the hydrolyzed samples. We injected these 3 kDa fractions into a mass spectrometer to obtain chromatographic profile (Figure S6). The chromatograms did not show a remarkable difference in the peak profiles, although certain peaks in QPF-PN/FL appear to be bigger. There is a need to further characterize the 2–5 kDa band in the Tricine gels to identify the peptides.

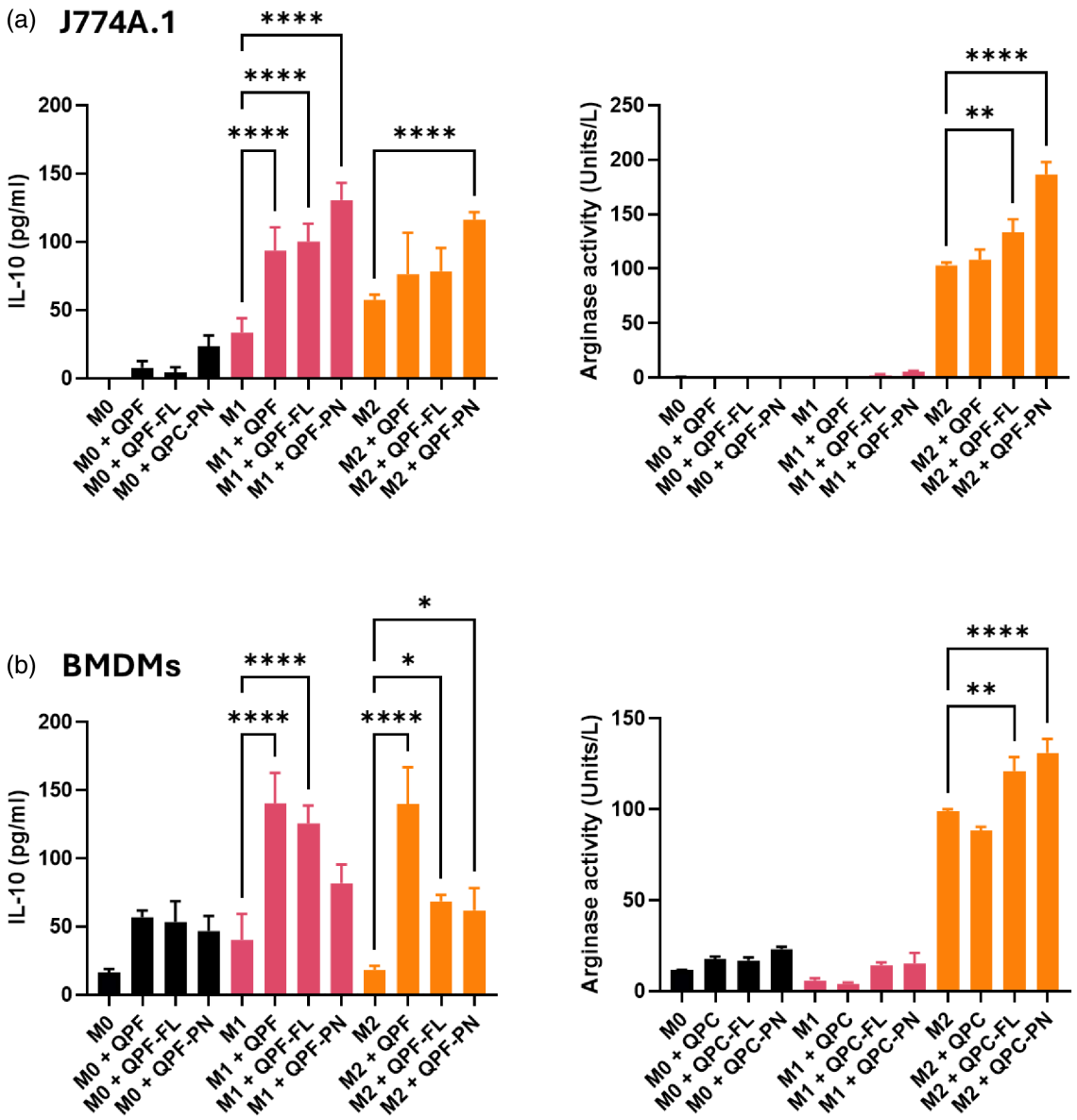

FIGURE 4 Effect of QPF and its hydrolysates on IL-10 and arginase activity. IL-10 production and arginase activity in J774A.1 (a) and BMDMs (b) upon polarization into M0 (naïve), M1-like, or M2-like phenotypes in the presence or absence (control) of water extracts (2 mg·mL^{-1}) QPF, QPF-FL, or QPF-PN. Data are expressed as mean values ± SD (one-way ANOVA with Tukey's post-test) from triplicate experiments. Asterisks indicate statistically significant differences (****$p < 0.0001$; ***$p < 0.001$; **$p < 0.01$) of treatments with the extracts compared to corresponding untreated controls. BMDM, bone marrow-derived macrophages; QPF, quinoa protein-enriched fraction; QPF-FL, QPF hydrolyzed by Flavourzyme; QPF-PN, QPF hydrolyzed by Papain.

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The amino acid profiles of the water-soluble extracts used to treat the cells may offer further explanation on the difference in the immunomodulatory effects observed between control and hydrolyzed samples. Indeed, amino acids with known immunomodulatory effects (Zheng et al., 2023), including leucine, arginine, methionine, glycine, and lysine were significantly higher in QPF-FL and QPF-PN compared to the control (Table 2). It is therefore rational to argue that the observed inhibitory effects on M1-like cytokine production and the enhancement of M2-like polarizing effects can be a collective or synergistic effect of immunomodulatory peptides and amino acids that are present in higher concentrations in the hydrolyzed samples. In particular, Leucine (Leu) was 3- to 4-fold higher in both hydrolyzed samples compared to the control. A recent study demonstrated that Leu could decrease LPS-induced M1-like polarization (inhibited IL-6, TNF-α) and promoted IL-4-mediated M2-like polarization (upregulated Arg-1 activity and Arg-1 protein expression) in BMDMs (Yan et al., 2024). Our results are consistent with their findings.

Non-nutrient components potentially present in QPF-FL or QPF-PN may also contribute to the observed modulation of macrophage polarization. Quinoa contains a range of polyphenols, for example,

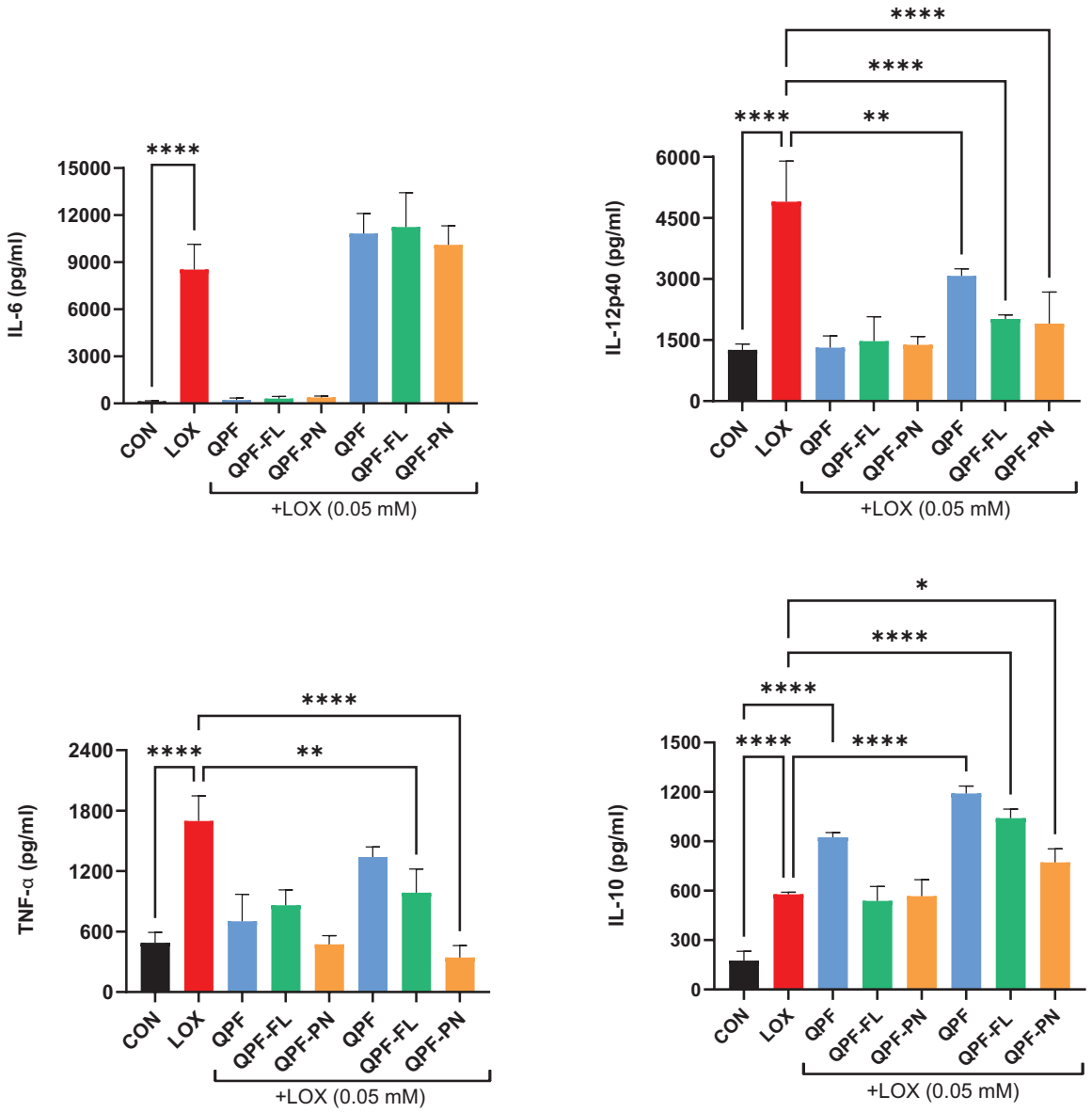

FIGURE 5 Cytokine production in BMDCs upon stimulation with loxoribine (LOX) in the presence or absence (control) of water extracts from QPF, QPF-FL, or QPF-PN. Data are expressed as mean values ± SD (one-way ANOVA with Tukey's post-test) from triplicate experiments. Asterisks indicate statistically significant differences (****$p < 0.0001$; **$p < 0.01$) of treatments with the extracts compared to corresponding untreated controls. BMDC, bone marrow-derived dendritic cells; ns, no significant difference; QPF, quinoa protein-enriched fraction; QPF-FL, QPF hydrolyzed by Flavourzyme; QPF-PN, QPF hydrolyzed by papain.

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flavonoids and phenolic acids that may be covalently conjugated to rod-shaped proteins or to cell wall structural substances or sugar moieties of cellulose, hemicellulose, lignin, or pectin (Song et al., 2022). There is evidence that phenolic compounds can be released during enzymatic hydrolysis to increase antioxidant and immunoregulatory effects of resulting protein hydrolysates (Islam et al., 2023). For example, enzymatic hydrolysis of quinoa protein isolates remarkably increased the antioxidant activities of the protein hydrolysates, which was accompanied by significant increase in total phenolic contents (de Carvalho et al., 2024). Various other studies have also shown release of phenolics from protein-rich fractions after enzymatic hydrolysis whereby bound phenolics (glycosides) can be released into free phenolics (aglycones) (Liu et al., 2017; Pimentel et al., 2020; Ribeiro et al., 2013; Sun et al., 2021).

QPFH drives macrophage polarization into the anti-inflammatory M2-like phenotype

QPF and QPF-FL/PN induced production of IL-10 in both the inflammatory M1-like and anti-inflammatory M2-like states exposed to similar concentrations of the samples (Figure 4). For both M2-like J774 or M2-like BMDMs, QPF had no boosting effect, but both QPF-FL and QPF-PN significantly increased arginase activity vs. the M2-like control. Arginase activity was induced significantly by the hydrolyzed samples in both M1/M2-like states, but this effect is more significant in M2 (Figure 4). It is notable that QPF-PN had a more potent boosting effect than QPF-FL. The induction of IL-10 and arginase activity is a characteristic of anti-inflammatory/pro-resolving M2-like macrophages, which permits resolution of inflammation and tissue repair (Viola et al., 2019). The increase in arginase

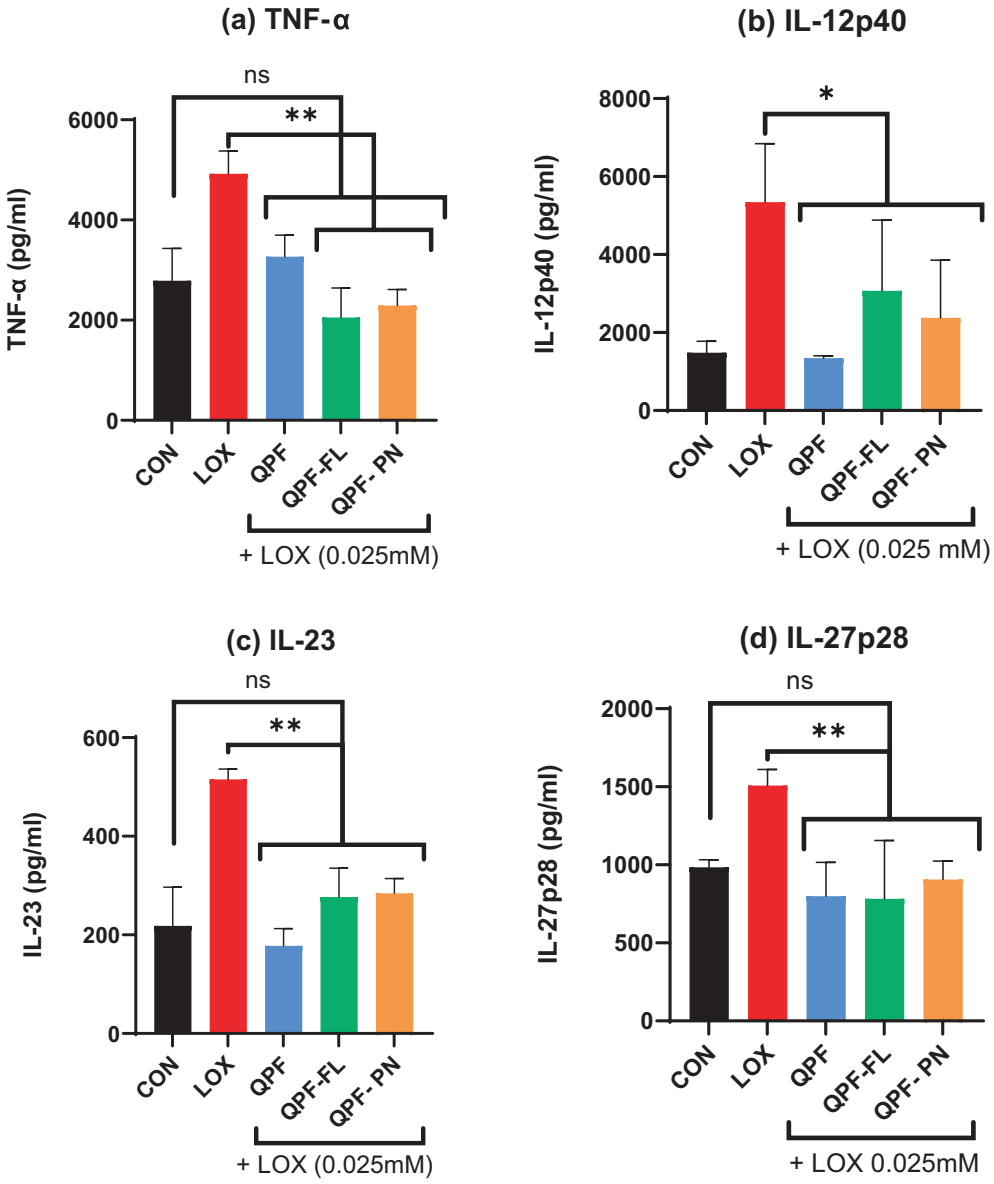

FIGURE 6 Impact of QPF and its hydrolysates on cytokine production during BMDC maturation in the presence of LOX. Data are expressed as mean values ± SD (one-way ANOVA with Tukey's post-test) from triplicate experiments. Asterisks indicate statistically significant differences (**$p < 0.01$; *$p < 0.05$) of treatments with the extracts compared to corresponding untreated controls. BMDC, bone marrow-derived dendritic cells; ns, no significant difference; QPF, quinoa protein-enriched fraction; QPF-FL, QPF hydrolyzed by Flavourzyme; QPF-PN, QPF hydrolyzed by papain.

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activity results in the production of polyamines and collagen and favors tissue remodeling and wound healing (Mantovani et al., 2013). To our knowledge, this is the first study that provides in vitro evidence that QPF-FL/PN could promote a switch in macrophage activation from the pro-inflammatory to the anti-inflammatory M2-like subtype.

Hydrolyzed QPF modulates cytokine production during BMDC maturation

The observed influence on the cytokines and surface antigen molecules suggests that a similar impact can be observed in DCs. To expand our investigation, we used a known TLR7 agonist loxoribine (LOX) to activate JAWSII cells and in the BMDC maturation experiment. All samples inhibited TNF-α and IL-12p40 in LOX-activated BMDCs, with QPF-FL and QPF-PN showing more potent effects (Figure 5). Interestingly, a similar boosting effect of the samples on IL-10 as seen in macrophages was observed further confirming the IL-10 upregulating effect of quinoa. In a separate experiment where immature BMDCs were exposed to lower concentrations of LOX and the samples over a 7-day course of maturation, the same inhibitory effect of all samples on TNF-α and IL-12p40 was observed (Figure 6). In addition, IL-23 and IL-27p28 were also significantly inhibited by all samples, however this time the hydrolysates no longer displayed superior potency over the unhydrolyzed sample. To our knowledge, this is the first in vitro study showing the potential inhibitory effects of QPF and its hydrolysates on TLR activation-mediated activation of DCs.

QPF but not its hydrolysates modulates the expression of MHCI

Aside from the production of high levels of inflammatory cytokines, M1-like macrophages express surface antigen molecules, including CD80, CD86, and major histocompatibility complex class I or II receptor (MHC1/MHCII) and promote Th1 responses (Martinez & Gordon, 2014; Mosser & Edwards, 2008). Our flow cytometry data showed that only QPF (unhydrolyzed) showed marked inhibitory effect on MHCI expression in M1-like J774 cells (Figure 7). All three samples had negligible effects on CD80 and CD86 (Figure S5). The M1 polarizing agents (IFN-γ + LPS) did not influence MHCII expression vs. the unstained and naïve controls (data not shown). Therefore, the effect of the samples on MHCII was not investigated. We carried out the same experiments in JAWII DCs to test if we could observe the same effect on QPF on MHCI. We used LOX (TLR7) and LPS (TLR4) as TLR agonists. LOX did not increase the MFI for MHCI vs. control, but a significant decrease in MFI (29%) was observed in cells exposed to LOX + QPF compared to LOX alone (Figure 8). LPS caused a significant increase in MHCI MFI vs. control, while the LPS + QPF treatment decreased the MHCI MFI by 25% compared to LPS alone. Taken together, our results revealed two important observations: (1) that the effect of QPC antigen molecule-specific, particularly MHCI and (2) that the modulatory effect of QPF-FL or QPF-PN on the surface antigen molecules is generally muted compared to the unhydrolyzed protein. Given that there are virtually no other studies reporting on the effect of quinoa on surface antigen molecules, the interesting trends we

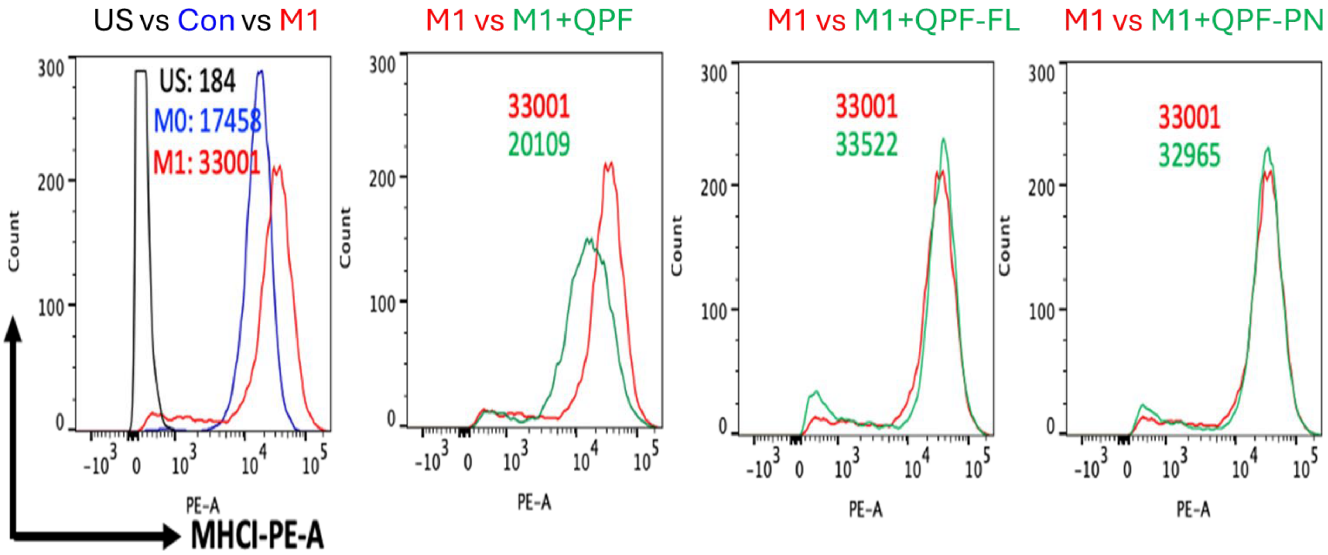

FIGURE 7 Effect of QPF and its hydrolysates on the expression MHCI on M1-like polarized macrophages. Single-cell suspensions were stained with a fluorochrome-conjugated specific antibody and Flow cytometric analysis was carried out on 7-AAD-negative cells (to exclude dead cells). QPF, quinoa protein-enriched fraction; US, unstained control.

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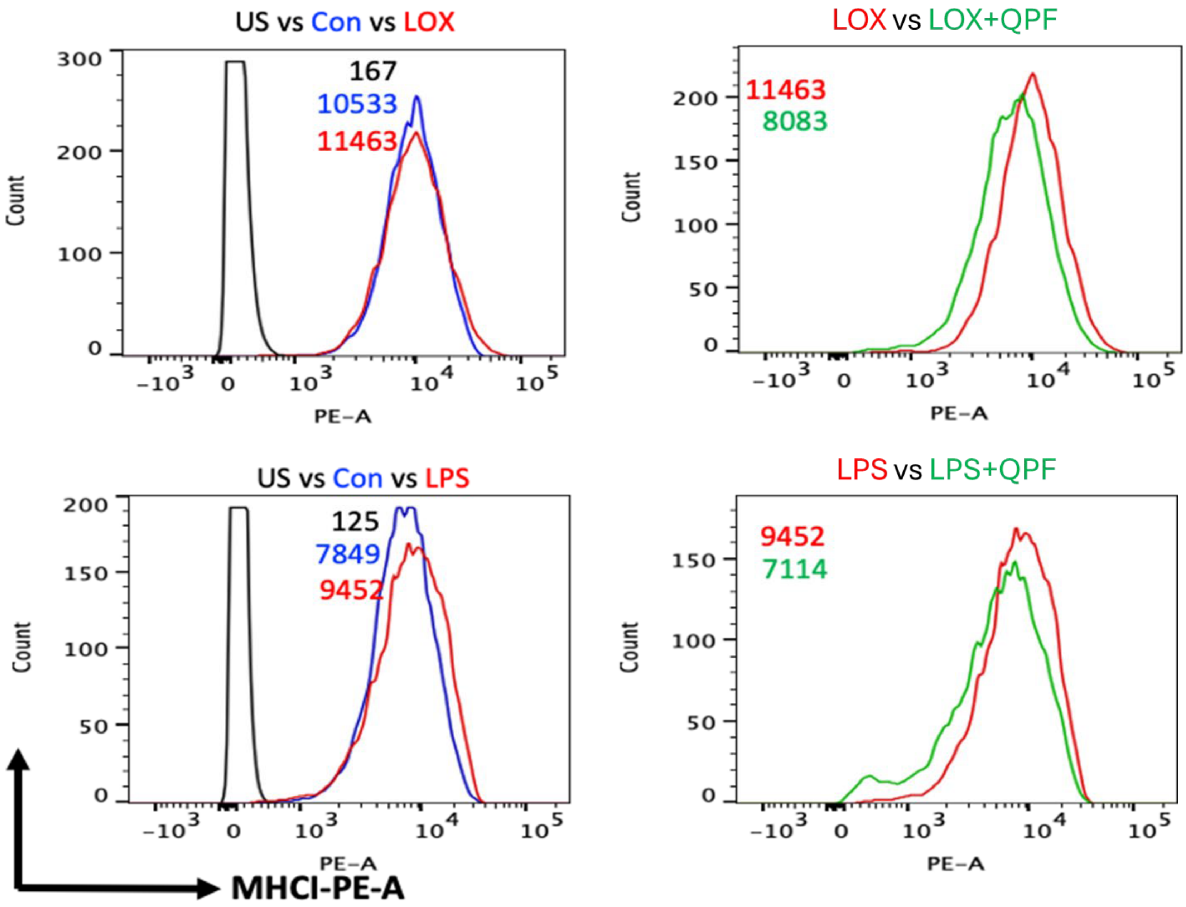

FIGURE 8 Effect of QPF on the expression of MHCI on LOX- and LPS-activate dendritic cells. Single-cell suspensions were stained with fluorochrome-conjugated specific antibodies and Flow cytometric analysis was carried out on 7-AAD-negative cells (to exclude dead cells). LOX, loxoribine; LPS, lipopolysaccharide; QPF, quinoa protein-enriched fraction; US, unstained control.

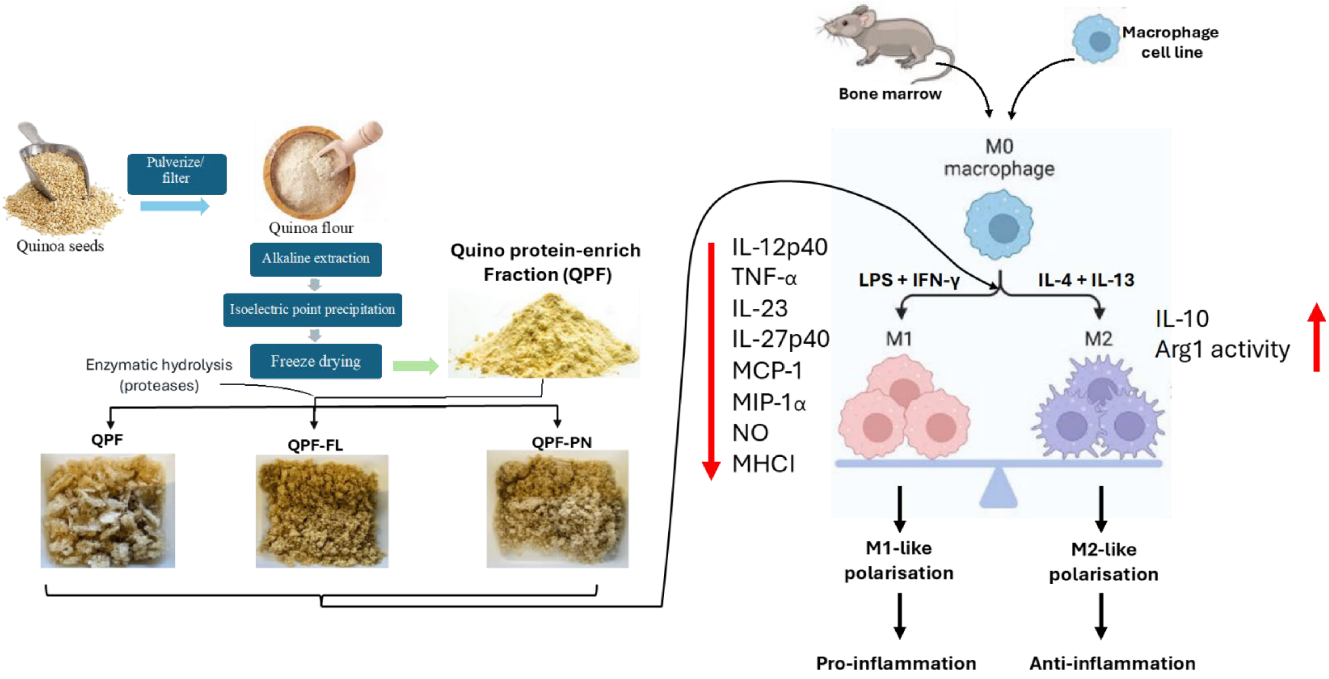

FIGURE 9 Hydrolyzed QPF inhibits M1-like polarization and promotes M2-like polarization of BMDMs and J774 macrophages. BMDM, bone marrow-derived macrophages; QPF, quinoa protein-enriched fraction; QPF-FL, QPF hydrolyzed by Flavourzyme; QPF-PN, QPF hydrolyzed by papain.

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observed warrant further investigations using in vitro (primary cells) and in vivo models.

CONCLUSION

In conclusion, QPF and the enzymatic hydrolysates, QPF-FL and QPF-PN, had differential modulatory effects on macrophage polarization and DC activation in vitro. The present work demonstrates that hydrolyzed quinoa protein fractions are more potent in reducing the inflammatory responses in M1-like macrophages and upregulating arginase activity and the production of IL-10 in both M1-like and M2-like macrophages compared to unhydrolyzed proteins (Figure 9). These results suggest that hydrolyzed quinoa protein could influence macrophage polarization to favor the anti-inflammatory M2-like type. In addition, the hydrolyzed proteins have shown the ability to inhibit pro-inflammatory responses in DCs. One major limitation of our study is the use of only one variety of quinoa; therefore, our results will need to be carefully interpreted as there are different varieties of quinoa being grown and consumed in different countries. Another limitation of this study was the 3 h enzymatic hydrolysis used to generate the protein hydrolysates. The duration of protein hydrolysis can be a critical factor that could influence the immunomodulatory effects of the hydrolysates. Taken together, our results showed that enzymatic hydrolysis of quinoa protein using food-grade proteases may confer enhanced immunomodulatory properties to the protein hydrolysates, suggesting that QPF-FL or QPF-PN can be a potential immunomodulatory protein-based functional ingredient. Future studies should investigate the identities of peptides in the 3 kDa fraction and verify whether these peptides, indeed, are responsible for the M2-like polarizing effects of the water-soluble extracts from hydrolysed quinoa.

AUTHOR CONTRIBUTIONS

Conceptualization: R.T., M.C., and C.L. *Conduct of experiments*: R.T., D.B., E.Q., and D.F. *Writing—original draft preparation*: R.T., D.B. *Writing—review and editing*: R.T., C.L., and M.C. *Project administration*: R.T. and C.L. *Funding acquisition*: R.T, C.L., and M.C. All authors have read and agreed to the published version of the manuscript.

ACKNOWLEDGMENTS

We thank Dr. Nicholas Michael of the Chemical Analysis Facility of University of Reading for his technical assistance with the LC–MS analysis of peptide fractions.

FUNDING INFORMATION

R.T. received funding for this project from the European Union's Horizon 2020 Research and Innovation Programme under the Marie Skodowska-Curie Grant Agreement MF20210255 and was further supported by seed funding for new lecturers of the Department of Food and Nutritional Sciences, University of Reading. C.L. and D.F. are supported by Enterprise Ireland (grant number: TC20180025).

CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflict of interest.

DATA AVAILABILITY STATEMENT

The datasets generated during and/or analyzed in the current study are available from the corresponding author on reasonable request.

ETHICS STATEMENT

No humans were used in the research for this paper. The care, treatment, and experiments involved in this study were approved by the Research Ethics Committee (REC) of Dublin City University (Approval ID: DCUREC/2023/187).

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Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Tocmo R, Balagiannis D, Quinn E, Finnegan D, Collins M, Loscher CE. Protein hydrolysates from quinoa (*Chenopodium quinoa* Willd.) modulate macrophage polarization and the expression of surface antigen molecules. Sust Food Prot. 2024. https://doi.org/10.1002/sfp2.1042

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