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Effect of β-glucans on rainbow trout (*Oncorhynchus mykiss*) IgM$^+$ B cells

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ABSTRACT

β-glucans are carbohydrates present in the cell wall of many fungi, which are often used as immunostimulants in feeds for farmed species. Their capacity to activate innate immune responses directly acting on innate cell populations has been widely documented in fish. However, whether they can affect the functionality of adaptive immune cells has been scarcely explored. In this context, in the current work, we have determined the effects of β-glucans on rainbow trout blood IgM$^+$ B cells in the presence or absence of 2,4,6-trinitrophenyl hapten conjugated to lipopolysaccharide (TNP-LPS), a model antigen. For this, rainbow trout peripheral blood leukocytes were incubated with different doses of β-glucans or media alone in the presence or absence of TNP-LPS for 48 h. The size, levels of expression of surface MHC II, antigen processing and phagocytic capacities and proliferation of IgM$^+$ B cells were then studied by flow cytometry. The number of IgM-secreting cells in the cultures was also estimated by ELISpot. β-glucans significantly decreased the levels of surface MHC II expression and the antigen processing capacities of these cells, especially in the presence of TNP-LPS, while they increased their phagocytic activity. On their own, β-glucans slightly activated the proliferation of IgM$^+$ B cells but reduced that induced by TNP-LPS. In contrast, β-glucans significantly increased the number of cells secreting IgM in the cultures. This effect of β-glucans on the IgM-secreting capacity of B cells was also confirmed through a feeding experiment, in which the IgM-secreting capacity of blood leukocytes obtained from fish fed a β-glucan-supplemented diet for one month was compared to that of leukocytes obtained from fish fed a control diet. Altogether, these findings contribute to increase our knowledge regarding the effects of β-glucans on fish adaptive responses.

1. Introduction

β-glucans are polysaccharides that serve as the primary structural component of the cell wall of some cereal plants (such as oats and barley), algae (including *Laminaria* sp.), fungi (such as *Saccharomyces cerevisiae* and *Cryptococcus neoformans*) and bacteria (such as those in the *Rhizobiaceae* family) [1,2]. β-glucans from different sources have in common that they are polymers of repeating units of D-glucose, linked by β-glycosidic bonds, yet they vary in molecular weight, shape and structure, with these factors having an important impact on their immune effects [2].

The immunostimulatory activity of β-glucans has been broadly demonstrated in many fish species [1,3], and for this reason, these molecules are sometimes included as components of functional feeds for farmed species. As a consequence of their immunostimulatory effects, feeding of β-glucan-enriched diets for several weeks has been shown to increase the survival rate of diverse fish species challenged with bacteria [4,5], virus [6,7] or even parasites [8,9]. However, this is not a universal effect, and in some cases such as that of Atlantic salmon (*Salmo salar*) exposed to sea lice (*Lepeophtheirus salmonis*), higher infestations have been obtained in fish treated with β-glucans [10]. Interestingly, β-glucans have not only been shown to stimulate the innate immune system but have also been demonstrated to generate positive effects on growth, development, nutrient absorption and intestinal balance [11], also helping reduce mortalities under stress conditions [12].

β-glucans are recognized by immune cells as pathogen-associated molecular patterns (PAMPs), hence triggering in these cells a pro-inflammatory response that includes secretion of cytokines and chemokines and production of reactive oxygen species [13,14]. The prototypical receptor for β-glucans in mammals is dectin-1 [15]. Other molecules seen to mediate the recognition of β-glucans in mammals include the complement receptor type 3 (CR3), lactosylceramide or

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additional scavenger receptors [14,16,17]. Interestingly, different innate leukocyte subsets seem to express a different range of these receptors capable of recognizing β-glucans, and as a consequence of this different pattern of receptor expression, β-glucans provoke different effects in them [18].

Dectin-1 seems absent from all fish genomes explored to date [19], yet different fish species have been shown to contain homologue genes to mammalian CR3 [20]. However, the role of the coded protein in the recognition of β-glucans in fish remains to be demonstrated. Interestingly, some studies have pointed to TLR2 by itself or in association with other receptors as capable of sensing β-glucans [19]. Again, TLR2 is expressed in most fish species, yet the capacity of fish TLR2 to detect β-glucans has only been suggested by indirect studies [21]. Nonetheless, in fish, the capacity of β-glucans to up-regulate the production of reactive oxygen species or nitric oxide (NO) [22], the phagocytic activity [23] or production of neutrophil extracellular traps (NETs) [24] by innate cells has been clearly demonstrated.

It is well known that innate immune mechanisms triggered during the early onset of antigen recognition strongly condition the adaptive immune responses generated, being this the basis for vaccine adjuvants [25]. Hence, the general mechanism of action of an important group of adjuvants is that they are recognized as PAMPs by pattern recognition receptors (PRRs) present on the immune cells and then initiate an inflammatory response that leads to an increased activation of the adaptive immune response. It is for this reason, that β-glucans have been widely explored as adjuvants both in mammals [26] and fish [5,27,28], with mostly positive effects. However, whether these adjuvant effects are also a consequence of direct effects on cells of the adaptive immune system is not well known to date, since the potential of β-glucans as direct stimulators of cells of the adaptive immune system such as B cells has only been scarcely explored [29].

In this context, this work aimed to investigate the effects of β-glucans on fish B cells, using rainbow trout (*Oncorhynchus mykiss*) as a model. For this, we performed several *in vitro* assays using blood leukocytes from naive rainbow trout. These cells were exposed to various concentrations of β-glucans in the presence or absence of a model antigen, 2,4,6-trinitrophenyl hapten conjugated to lipopolysaccharide (TNP-LPS), known to strongly activate trout IgM^+ B cells [30]. We then evaluated the response of IgM^+ B cells in the culture, determining their size, IgM-secreting capacity, levels of MHC II surface expression, antigen-processing and phagocytic abilities and proliferative capacities. Additionally, we conducted an *in vivo* experiment in which fish were fed with a β-glucan- supplemented diet for 1 month and determined the effects on IgM^+ B cells. The results generated contribute to a better understanding of the mechanisms of action of β-glucans in fish, and will be useful for designing prophylactic strategies involving their use in aquaculture.

2. Material and methods

2.1. Experimental fish

Non-stimulated rainbow trout (*Oncorhynchus mykiss*) of approximately 100 g obtained from Piscifactoria Cifuentes (Cifuentes, Guadalajara, Spain) were used to obtain blood leukocyte populations and perform the *in vitro* analyses. Fish were maintained at the animal facilities of the Animal Health Research Center (CISA-INIA-CSIC, Spain) in an aerated recirculating water system at 16 °C, with a 12:12 h light: dark photoperiod. These fish were fed twice a day with a commercial diet (Skretting) and were acclimatized to laboratory conditions for at least 2 weeks before any experimental procedure. During this period, no clinical signs of disease were ever observed.

Rainbow trout of approximately 35 g obtained from Piscifactoria Riomundo (Albacete, Spain) were used in the *in vivo* feeding experiment. These fish were maintained at the Aquaculture Research Center (ITACyL, Segovia, Spain) in the same conditions as described above. Before the start of the feeding trial, fish were acclimatized to laboratory conditions for 4 weeks. During this period, all fish were fed the control diet later used in the experiment and no clinical signs of disease were ever observed.

All the experiments described above comply with the Guidelines of the European Union Council (2010/63/EU) for the use of laboratory animals and were approved by the INIA Ethics Committee (PROEX 065.3/21).

2.2. Leukocyte isolation and in vitro stimulation with β-glucans

Fish were euthanized by anesthetic overdose using a water bath with approximately 150 ppm of benzocaine (Sigma). Blood was extracted with a heparinized needle from the caudal vein and diluted 10 times with Leibovitz's medium (L-15, Gibco) supplemented with 100 IU/ml penicillin, 100 mg/ml streptomycin (P/S), 10 units/ml heparin and 2 % fetal calf serum (FCS) (all supplements obtained from Life Technologies). Blood cell suspensions were placed onto 51 % Percoll (GE Healthcare) cushions and were centrifuged at 400×*g* for 30 min at 4 °C, without brake. The interface cells were collected and washed with L-15 containing antibiotics and 2 % FCS. Counting and cell viability were then determined by trypan blue (Sigma-Aldrich) exclusion, and cells were resuspended in L-15 medium supplemented with P/S and 5 % FCS at a concentration of 2×10^6 cells/ml.

To evaluate the effect of β-glucans on blood B cells, we used commercial *S. cerevisiae* glucans (60 % purity) provided by Skretting. A stock solution was obtained diluting the β-glucans in L-15 medium without supplements. Afterwards, different volumes of the stock solution were added to total blood leukocytes incubated in L-15 medium containing P/S and 5 % FCS, immediately after their isolation, to obtain different final concentrations of this carbohydrate (0, 6, 30 and 60 μg/ml) in the cultures. These concentrations were chosen based on previous *in vitro* studies performed in the laboratory in which different β-glucans doses were tested as inducers of a pro-inflammatory transcriptional response in fish cell lines (Fig. S1). Cells in all conditions (supplemented or not with β-glucans) were either stimulated with 5 μg/ml of 2,4,6-trinitrophenyl hapten conjugated to lipopolysaccharide (TNP-LPS) or left unstimulated, following the protocol described before [30]. TNP-LPS was added, when required, immediately after isolation, together with the β-glucans. After 48 h of incubation at 20 °C, cells were analyzed by flow cytometry. The number of IgM-secreting cells in the cultures was also determined at this time point by ELISpot.

2.3. Flow cytometry analysis of the blood leukocytes stimulated with β-glucans

Blood leukocytes were stained with anti-trout IgM [1.14 monoclonal antibody (mAb) mouse IgG1 coupled to R-phycoerythrin (R-PE); 1 μg/ml] [31], anti-trout IgD [mAb mouse IgG1 coupled to allophycocyanin (APC); 5 μg/ml] [32] and anti-trout MHC II β-chain [mAb mouse IgG1 coupled to fluorescein isothiocyanate (FITC); 1.5 μg/ml] [33] diluted in staining buffer (phenol red-free L-15 medium supplemented with 2 % FCS and P/S) for 1 h in darkness at 4 °C. Following the incubation, cells were washed twice and resuspended in the staining buffer for their analysis in a FACS Celesta™ flow cytometer (BD Biosciences) equipped with BD FACSDiva software (BD Biosciences). The data obtained were analyzed using the FlowJo® v.10 software (FlowJo LLC, Tree Star). In all cases, the cell viability was checked using 4′,6-diamine-2' -phenylindole dihydrochloride (DAPI) at 0.2 μg/ml.

2.4. Analysis of antigen-processing capacity

The antigen-processing capacity of total IgM^+ B cells in leukocyte cultures was measured using the EnzChek protease Assay kit (Invitrogen). Briefly, blood leukocytes at a concentration of 2×10^6 cells/ml, seeded in 96-well plates (100 μl/well), were incubated in the presence or

absence of TNP-LPS (5 μg/ml) in media supplemented or not with the different concentrations of β-glucans as described above. After 48 h, cells were incubated with green fluorescent BODIPY DQ-CASEIN at 5 μg/ml for 1 h. BODIPY DQ-casein is a self-quenched form of fluorescently labelled casein commonly used to study protease-mediated antigen processing given that it exhibits bright green fluorescence upon proteolytic processing due to the released dye molecules [34]. Afterwards, the cells were washed with staining buffer and labelled with the anti-IgM mAb coupled to APC (1 μg/ml) for 30 min at 4 °C, washed again, and analyzed by flow cytometry as described above.

2.5. Phagocytic activity

To analyze the effect of β-glucans on the phagocytic capacity of IgM^+ B cells, blood leukocytes were seeded in 24-well plates at a cell density of 2×10^6 cells per well, in the presence or absence of TNP-LPS (5 μg/ml) in media supplemented or not with the different concentrations of β-glucans as described above. After 48 h, cells were collected and resuspended in L-15 medium without serum, and then incubated for 3 h at 20 °C with fluorescent beads (FluoSpheres R Microspheres, 1.0 μm, Crimson Red Fluorescent 625/645, 2 % solids; Thermo Fisher Scientific) at a cell:bead ratio of 1:10 as described before [35]. After the incubation period, cells were harvested by gently pipetting, and non-ingested beads were removed by centrifugation (100×*g* for 10 min at 4 °C) over a cushion of 3 % (weight/volume) BSA (Fraction V; Fisher Scientific) in PBS supplemented with 4.5 % (weight/volume) D-glucose (Sigma). Cells were then resuspended in staining buffer, labelled with anti-IgM-FITC (1.14) (1 μg/ml) and analyzed on a FACS Celesta flow cytometer. The data obtained were analyzed using the FlowJo® v.10 software. Cell viability was checked using DAPI as described above.

2.6. B cell proliferation assay

The Click-IT® EdU Alexa Fluor® 488 Flow Cytometry Assay Kit (Life Technologies) was used to measure the proliferation of total IgM^+ B cells following the manufacturer's instructions. For this, blood leukocytes were exposed to TNP-LPS (5 μg/ml) or not in media supplemented or not with the different concentrations of β-glucans. After 48 h of incubation at 20 °C, 1 μM EdU (5-ethynyl-2′-deoxyuridine) was added to all cultures, and the cells were incubated for an additional 24 h. At this point, the viability of the cells was determined using the LIVE/DEAD Fixable Near-IR Dead Cell Stain kit (Invitrogen), following the kit's specifications. Cells were then washed and stained with anti-IgM mAb coupled to R-PE (1 μg/ml) for 30 min at 4 °C. Cells were then fixed, permeabilized, and incubated with specific reagents to detect the incorporation of EdU into the DNA of proliferating cells following the manufacturer's instructions. Samples were then analyzed by flow cytometry as described above.

2.7. ELISpot

ELISpot was used to quantify the number of total IgM-secreting B cells in leukocyte cultures as previously described [35]. For this, ELISpot plates containing Inmobilon-P membranes (Millipore) were activated with 70 % ethanol for 30 s, coated with anti-trout IgM mAb at 2 μg/ml in PBS (phosphate buffered saline, Sigma Aldrich), and incubated overnight at 4 °C in agitation. To block non-specific binding to the membrane, plates were then incubated with 2 % BSA (bovine serum albumin, Sigma-Aldrich) in PBS for 2 h at room temperature (RT). Thereafter, leukocytes that had been incubated for 48 h in the presence or absence of TNP-LPS with the different concentrations of β-glucans were transferred to pre-coated ELISpot plates. After 24 h at 20 °C, cells were washed away five times with PBS and plates blocked again with 2 % BSA in PBS for 1 h at RT. After blocking, biotinylated anti-trout IgM mAb was added to the plates at 1 μg/ml and incubated for 1 h at RT. Following additional washing steps with PBS, the plates were developed using streptavidin-HRP (Thermo Fisher Scientific) (100 ng/ml) for 1 h at RT, washed again with PBS and incubated with 3-amino 9-ethylcarbazole (Sigma-Aldrich) for 30 min at RT in the dark. The substrate reaction was stopped by washing the plates with tap water. Once the membranes were dried, they were digitally scanned and the number of spots in each well was determined using an AID iSpot Reader System (Autoimmun Diagnostika GMBH, Germany).

2.8. In vivo feeding experiment

To confirm the positive effects provoked by β-glucans on B cells obtained in the *in vitro* studies, a feeding experiment was performed to compare how a β-glucan supplemented diet affected the percentage of B cells in blood cultures and the number of IgM secreting cells. The control diet was formulated to fulfill the nutritional requirements for rainbow trout of this size, and was identical to the one used to feed the rainbow trout used to obtain blood leukocytes for the *in vitro* analysis. The other diet (β-glucans) was formulated to be identical to the control diet but supplemented with β-glucans at 0.1 % of feed weight, at the expense of wheat meal. Both diets were manufactured by Skretting (Norway).

For the experiment, fish were randomly assigned to identical tanks and received either the control or the β-glucans-supplemented diets. The fish were fed twice a day with each corresponding diet for one month at a rate of 2 % body weight per day. Thereafter, six fish from each group were sacrificed by benzocaine overdose. Blood was extracted with a heparinized needle from the caudal vein and blood leukocytes obtained as described above. Cells were immediately stained with specific mAb for flow cytometry analysis as described above or transferred to ELISpot plates pre-coated with anti-trout IgM mAb to quantify the number of total IgM-secreting B cells as also described above.

2.9. Statistical analysis

Data handling, statistical analyses and graphic representation were performed using GraphPad Software (GraphPad Prism v8.0.1, La Jolla California, USA). Statistical analyses were performed by two-way analysis of variance (ANOVA). The means were compared by Tukey multiple-range test two-way. The differences between the mean values were considered significant on different degrees, where * means $p \leq 0.05$, ** means $p \leq 0.01$, *** means $p \leq 0.001$.

3. Results

3.1. In vitro effect of β-glucans on the size of blood IgM + B cells

The effects of different concentrations of β-glucans (0, 6, 30 and 60 μg/ml), in the presence or absence of TNP-LPS, on IgM^+ cell populations present in blood leukocyte cultures were determined by flow cytometry. As established before, cells co-expressing IgM and IgD on the cell surface (IgM^+IgD^+ B cells) make up the majority of the IgM^+ B cell population among blood leukocytes (Fig. 1). Additionally, a small subset of IgM^+ B cells that have lost IgD expression and also reduced their levels of surface IgM (IgM^+IgD^- B cells) are also identified in these cultures (Fig. 1). Previous studies from our group have demonstrated that these cells correspond to B cells that have initiated a differentiation process towards plasmablast/plasma cells [36,37].

After verifying that β-glucans provoked no negative effects on the viability of these two B cell populations (data not shown), we analyzed the size of IgM^+IgD^+ and IgM^+IgD^- B cells in blood leukocyte cultures. Blood IgM^+IgD^+ B cells slightly increased their size in response to 60 μg/ml of β-glucans, in the absence of another stimulus (Fig. 2A and C). When cultures were stimulated with TNP-LPS, the size of IgM^+IgD^+ B cells increased as previously reported [30]. This increase was significantly higher when leukocyte cultures were treated with 30 or 60 μg/ml (Fig. 2A and C). In the case of IgM^+IgD^- B cells, the three concentrations of β-glucans tested provoked a significant increase in the size of these B

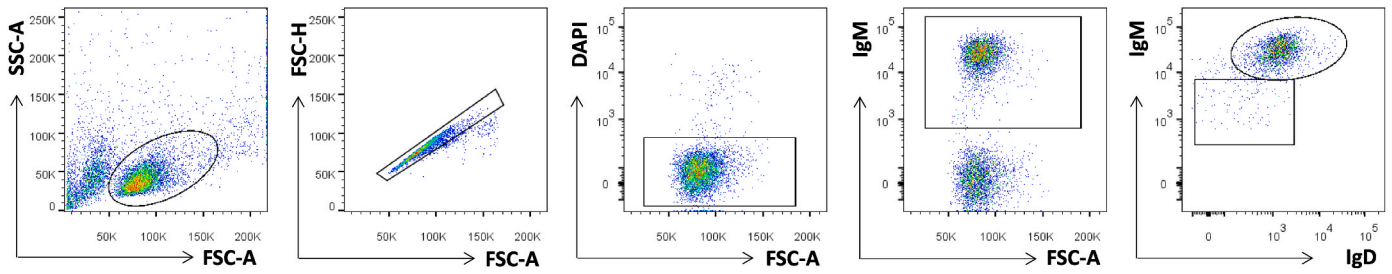


Fig. 1. Gating strategy for flow cytometry analysis. Total leukocytes were isolated from rainbow trout peripheral blood. FSC/SSC profiles including a defined gate for lymphoid cells are shown. FSC-H/FSC-A profiles within the lymphoid gate define singlets. DAPI-negative cells among singlets were gated to select alive cells. Total IgM^+ B cells were then selected and among them, two populations could be defined according to the levels of IgM and IgD surface expression, namely IgM^+IgD^+ and IgM^+IgD^- B cells.

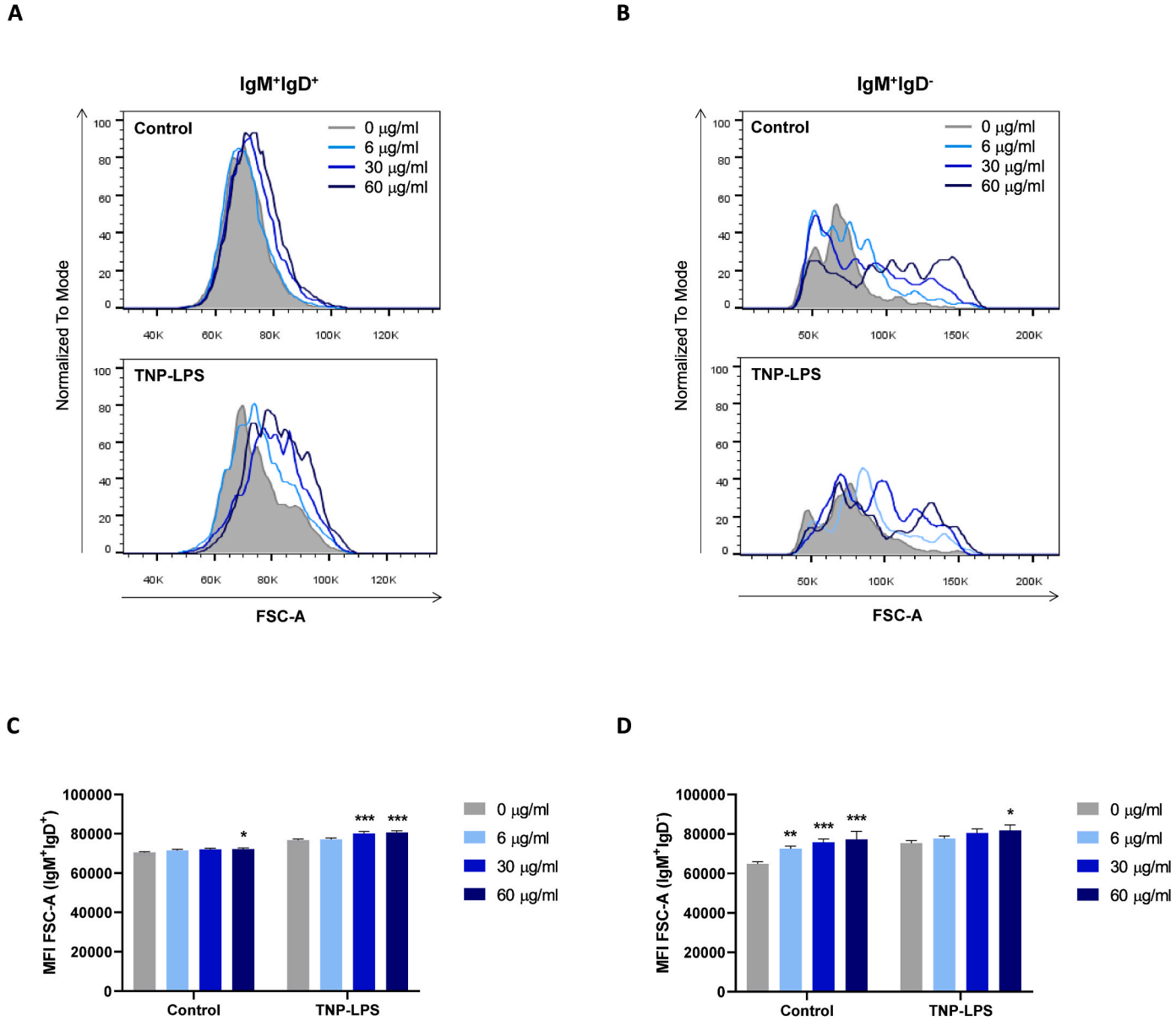


Fig. 2. *In vitro* effect of β-glucans on rainbow trout IgM + IgD^+ and IgM + IgD^- B cell size. Blood leukocytes were cultured with different concentrations of β-glucans in the presence or absence of TNP-LPS, as described in the Materials and Methods section. After 48 h of incubation at 20 °C, cells were labelled with anti-trout IgM and anti-IgD mAbs and analyzed by flow cytometry, following the gating strategy defined in Fig. 1. Representative histograms showing the size (forward side scatter, FSC) of IgM^+IgD^+ **(A)** and IgM^+IgD^- **(B)** B cells are shown, along with graphs presenting the mean fluorescence intensity (MFI) of FSC for IgM^+IgD^+ **(C)** and IgM^+IgD^- **(D)** B cells (mean +SEM; n = 13). Asterisks denote significantly different values between cultures supplemented with β-glucans and corresponding non-supplemented cultures (*$p \leq 0.05$, **$p \leq 0.01$, and ***$p \leq 0.005$).

cells in cultures non-stimulated with TNP-LPS (Fig. 2B and D). Again, this cell population increased its size in response to TNP-LPS and this increase was further increased in the presence of 60 μg/ml of β-glucans (Fig. 2B and D).

3.2. *In vitro effect of β-glucans on the levels of surface MHC II in blood IgM + IgD+ and IgM + IgD- B cells*

Given their professional antigen-presenting cell nature, B cells constitutively express MHC II on the cell surface and they can present antigens that they process in an MHC II context [38]. Thus, we next studied whether β-glucans affected the levels of MHC II surface expression of rainbow trout blood IgM^+IgD^+ and IgM^+IgD^- B cells by flow cytometry using a specific anti-trout MHC II antibody. As shown in the corresponding histograms and graphs (Fig. 3), the *in vitro* supplementation of blood leukocyte cultures with all tested concentrations of β-glucans significantly decreased the levels of surface MHC II on both IgM^+IgD^+ (Fig. 3A and C) and IgM^+IgD^- (Fig. 3B and D) B cells. This effect was observed both in cultures stimulated with TNP-LPS and in non-stimulated controls (Fig. 3). As previously established [30], TNP-LPS provoked a significant increase in the levels of MHC II surface expression on rainbow trout B cells (Fig. 3).

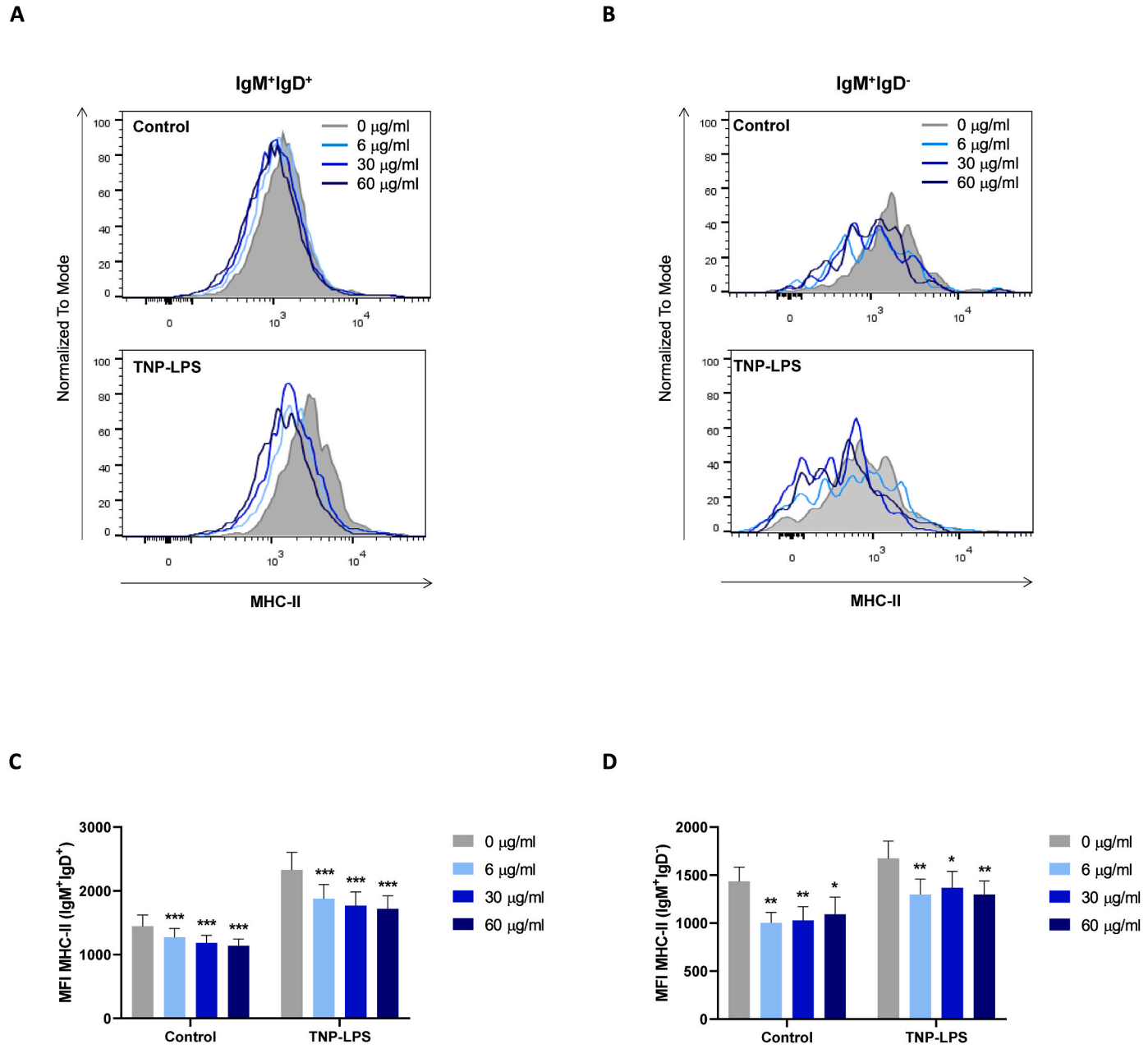


Fig. 3. *In vitro* effect of β-glucans on the levels of MHC II surface expression of IgM + IgD^+ and IgM + IgD^- B cells. Blood leukocytes were cultured with different concentrations of β-glucans in the presence or absence of TNP-LPS, as described in the Materials and Methods section. After 48 h of incubation at 20 °C, cells were labelled with anti-trout IgM, anti-IgD and anti-MHC II mAbs and analyzed by flow cytometry. Representative histograms showing MHC II expression levels on IgM^+IgD^+ **(A)** and IgM^+IgD^- **(B)** B cells are shown, along with graphs presenting the mean fluorescence intensity (MFI) of MHC II in IgM^+IgD^+ **(C)** and IgM^+IgD^- **(D)** B cells (mean +SEM; n = 15). Asterisks denote significantly different values between cultures supplemented with β-glucans and corresponding non-supplemented cultures (*$p \leq 0.05$, **$p \leq 0.01$, and ***$p \leq 0.005$).

3.3. *In vitro effect of β-glucans on the antigen-processing capacities of blood IgM + B cells*

Given the strong effects that β-glucans had on the levels of MHC II surface expression, we wanted to determine whether the capacity of B cells to process antigens was also affected in their presence. In this case, we studied the response of total IgM^+ B cells in the leukocyte cultures, analyzing their capacity to degrade DQ-CASEIN upon endocytosis. In concordance with the effects on MHC II, β-glucans at a concentration of 30 or 60 μg/ml significantly decreased the capacity of IgM^+ B cells to process DQ-CASEIN in TNP-LPS-stimulated cultures (Fig. 4).

3.4. *In vitro effect of β-glucans on the phagocytic capacities of blood IgM + B cells*

We next determined the effect of β-glucans on the phagocytic capacities of blood IgM^+ B cells on their own, or in the presence of TNP-LPS. Interestingly, in this case, β-glucans increased the phagocytic capacities of IgM^+ B cells in a dose-dependent fashion, both by themselves and when in the presence of TNP-LPS, reaching values significantly higher than those observed in non-stimulated controls with the two highest β-glucan doses (Fig. 5).

3.5. *In vitro effect of β-glucans on the proliferation of blood IgM + B cells*

We also analyzed if β-glucans promoted the proliferation of blood IgM^+ B cells on their own, or in the presence of TNP-LPS. On their own, the two highest β-glucan doses used induced a small but significant proliferation of IgM^+ B cells in blood leukocyte cultures (Fig. 6). As described before [30], stimulation with TNP-LPS for 3 days induced a strong proliferation of IgM^+ B cells in the leukocyte cultures (Fig. 6). This induced proliferation was decreased in the presence of any of the β-glucan doses tested (Fig. 6).

3.6. *In vitro effect of β-glucans on the number of IgM-secreting cells in leukocyte cultures*

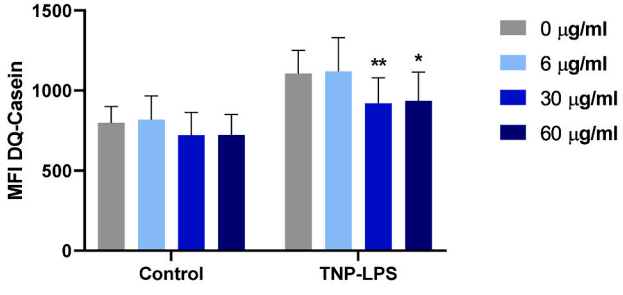
To establish whether β-glucans added *in vitro* to blood leukocyte cultures could affect the number of IgM-secreting cells, we performed an ELISpot assay using the cells that had been exposed to the different doses or β-glucans in the presence or absence of TNP-LPS. As shown in Fig. 7, the three doses of β-glucans tested were capable of increasing the number of IgM-secreting cells on their own. When leukocyte cultures were exposed to TNP-LPS, the number of IgM-secreting cells strongly increased as previously reported [30]. This induced IgM-secretion was further increased to levels significantly higher in the presence of 6 or 30 μg/ml β-glucans (Fig. 7).

3.7. *Effect of in vivo β-glucan supplementation on the survival of blood IgM + IgD+ and IgM + IgD- B cells and on the number of IgM-secreting cells*

Finally, we wanted to confirm the effects of β-glucans on rainbow trout B cells in a focused feeding *in vivo* experiment. For this purpose, fish were fed either a control diet or a diet supplemented with β-glucans. After 1 month of feeding, fish were sacrificed and blood leukocytes isolated to determine the percentage of IgM^+IgD^+ and IgM^+IgD^- B cells and the number of IgM-secreting cells by ELISpot. As shown in Fig. 8A, no differences were observed between the percentage of IgM^+IgD^+ isolated from fish fed the control and fish fed the β-glucan-supplemented diet. In contrast, the percentage of IgM^+IgD^- B cells significantly increased in fish fed with a β-glucan-supplemented diet (Fig. 8A). Interestingly, we determined the number of IgM-secreting cells by ELISpot analysis and observed that blood leukocyte cultures obtained from fish fed the β-glucan-enriched diet had a significantly higher number of IgM-secreting cells compared to cultures obtained from fish fed the control diet (Fig. 8B).

4. Discussion

β-glucans have been revealed as effective adjuvants in some



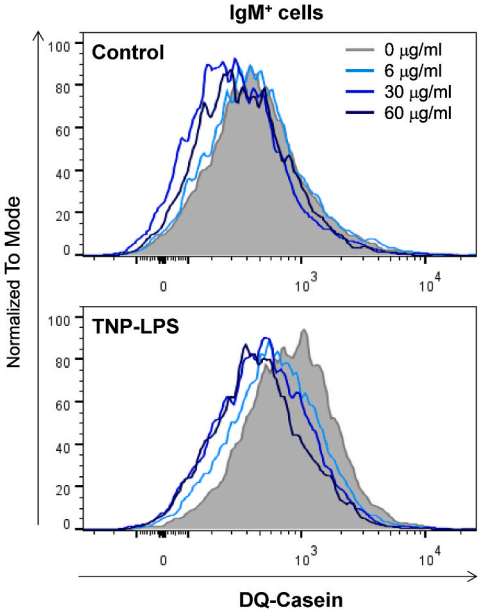


Fig. 4. *In vitro* effect of β-glucans on the antigen processing capacities of IgM + B cells. Blood leukocytes were cultured with different concentrations of β-glucans in the presence or absence of TNP-LPS, as described in the Materials and Methods section. After 48 h of incubation at 20 °C, cells were incubated with DQ-CASEIN and labelled with an anti-trout IgM mAb for flow cytometry analysis. Representative histograms are shown, along with a graph presenting DQ-CASEIN mean fluorescence intensity (MFI) values of IgM^+ B cells (mean +SEM; n = 8). Asterisks denote significantly different values between cultures supplemented with β-glucans and corresponding non-supplemented cultures (*$p \leq 0.05$ and **$p \leq 0.01$).

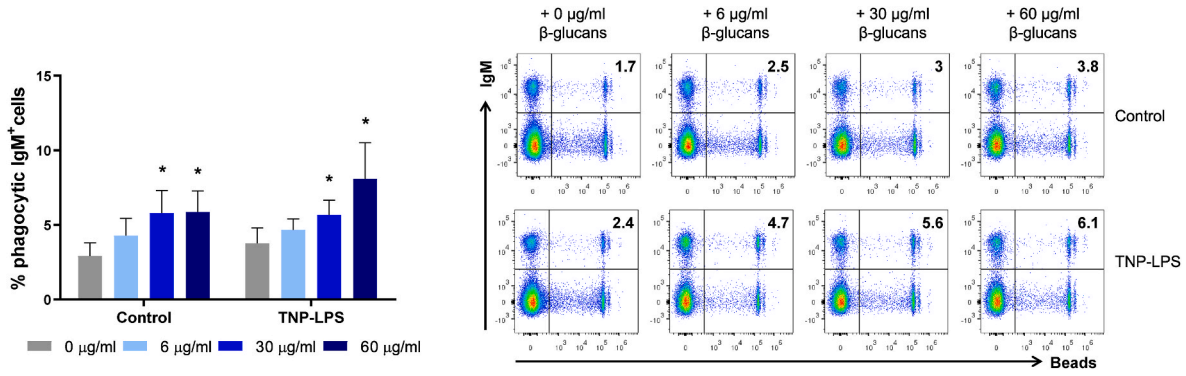


Fig. 5. *In vitro* effect of β-glucans on the phagocytic capacities of IgM + B cells. The effect of β-glucans on the phagocytic capacity of IgM^+ B cells was determined after incubating blood leukocyte cultures with different concentrations of β-glucans in the presence or absence of TNP-LPS, as described in the Materials and Methods section. After this time, cells were incubated with Crimson Red fluorescent beads (1 µm diameter) at a ratio of 1:10 (cell/beads) for a further 3 h at 20 °C. Non-ingested beads were removed by centrifugation over a cushion of 3 % BSA supplemented with 4.5 % D-glucose. Cells were then stained with anti-IgM mAb conjugated to FITC and analyzed by flow cytometry. The percentage of phagocytic IgM^+ B cells was then calculated. Representative dot plots are shown along with graphs presenting the percentage of phagocytic IgM^+ cells (mean +SEM; n = 6). Asterisks denote significantly different values between cultures supplemented with β-glucans and corresponding non-supplemented cultures (*$p \leq 0.05$).

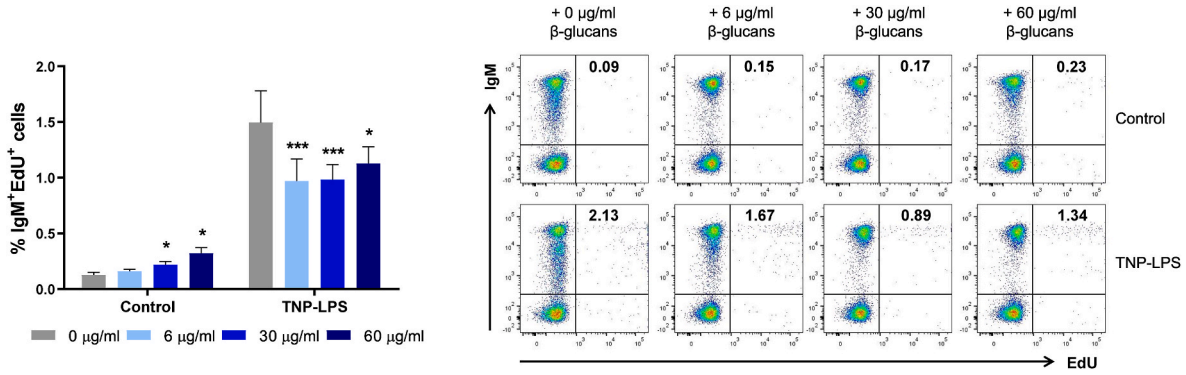


Fig. 6. *In vitro* effect of β-glucans on B cell proliferation. The lymphoproliferative effects of β-glucans were determined after incubating blood leukocyte cultures with different concentrations of β-glucans in the presence or absence of TNP-LPS, as described in the Materials and Methods section. After this time, cells were labelled with EdU (1 µM) and incubated for a further 24 h. Thereafter, cells were stained with anti-IgM mAb conjugated to APC. The percentage of IgM^+ B cells with incorporated EdU (proliferating IgM^+ B cells) within the total IgM^+ B cell population was then determined. Representative dot plots are shown along with graphs presenting the percentage of IgM^+EdU^+ cells within the IgM^+ cell population (mean +SEM; n = 13). Asterisks denote significantly different values between cultures supplemented with β-glucans and corresponding non-supplemented cultures (*$p \leq 0.05$ and ***$p \leq 0.005$).

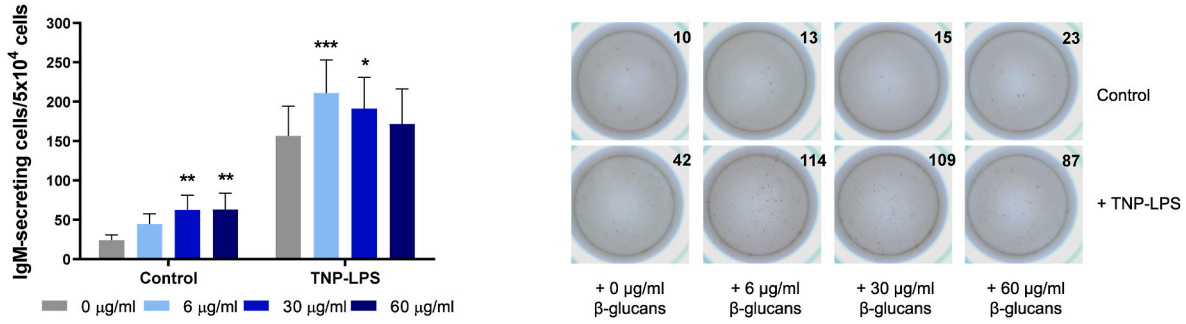


Fig. 7. *In vitro* effect of β-glucans on the number of IgM-secreting cells in blood leukocyte cultures. Blood leukocytes were cultured with different concentrations of β-glucans in the presence or absence of TNP-LPS, as described in the Materials and Methods section. After 48 h of incubation at 20 °C, the cells were transferred to ELISpot plates, previously coated with specific anti-IgM mAb (2 µg/ml), and incubated for a further 24 h. At this point, cells were washed away and a biotinylated anti-trout IgM mAb used to determine the number of cells secreting IgM. Wells from a representative fish are shown along with a graph representing the mean number of IgM-secreting cells per 5×10^4 cells (mean +SEM; n = 9). Asterisks denote significantly different values between cultures supplemented with β-glucans and corresponding non-supplemented cultures (*$p \leq 0.05$, **$p \leq 0.01$ and ***$p \leq 0.005$).

vaccination models in both mammals [26] and fish [5,27,28]. This adjuvant potential has always been attributed to the effect that β-glucans have on cells of the innate immune system [5,27,28], not investigating in depth the possible effects that β-glucans could have on cells of the adaptive immune system, such as B cells. In this context, in the current study, we have investigated the effects that β-glucans have on fish IgM^+

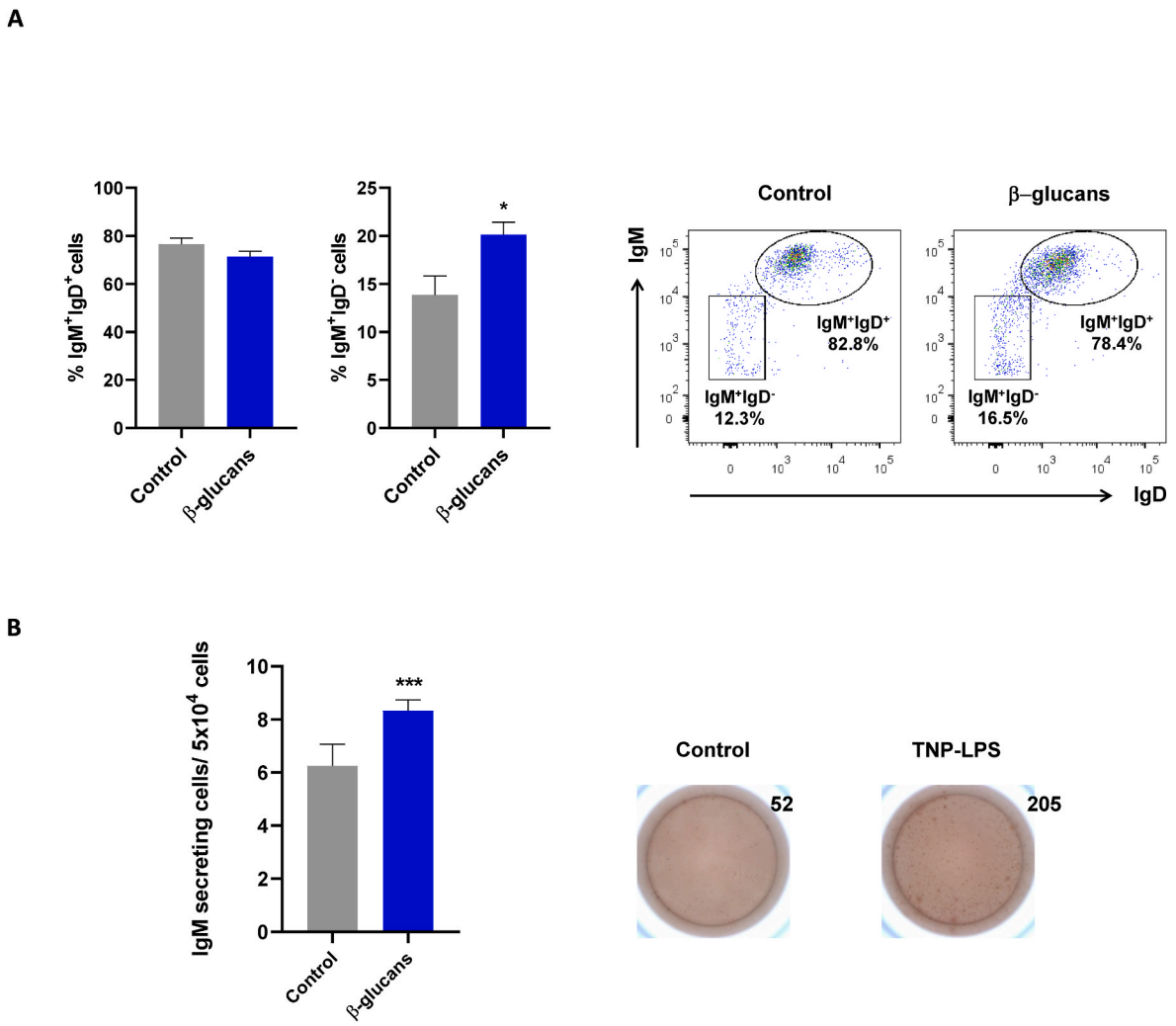


Fig. 8. Effect of a diet supplemented with β-glucans on IgM + B cell populations in blood. Rainbow trout were fed with a control diet or a diet supplemented with β-glucans for 30 days. After that time, fish were sacrificed and blood leukocytes isolated. **(A)** Blood leukocytes were labelled with anti-trout IgM and anti-trout IgD mAbs and analyzed by flow cytometry. Representative dot plots are shown along with graphs presenting the percentages of IgM^+IgD^+ and IgM^+IgD^- B cells in blood cultures (mean +SEM; n = 6). **(B)** Blood leukocytes were plated in ELISpot plates previously coated with a specific anti-IgM mAb (2 μg/ml) and incubated for 24 h. After that time, cells were washed away and a biotinylated anti-trout IgM mAb used to determine the number of cells secreting IgM. Wells showing spot-forming cells from a representative fish are shown along with a graph representing the mean number of IgM-secreting cells per 5×10^4 cells (mean +SEM; n = 6). Asterisks denote significant differences between values obtained in cultures obtained from fish fed a control diet and cultures obtained from fish fed the β-glucan-supplemented diet (*$p \leq 0.05$ and ***$p \leq 0.005$).

B cells, important elements of the fish adaptive immune system, using the rainbow trout as a model. We have not exclusively sorted IgM^+ B cells to conduct these studies, but instead used total blood leukocyte populations. Nonetheless, we have specifically focused on how these cells are specifically affected by β-glucans in the cultures and how these molecules affect the response of B cells to another stimulus such as TNP-LPS. Of course, we cannot rule out that these effects are a consequence of β-glucans inducing the production of cytokines or other stimulatory molecules on innate cells present in these cultures, and these molecules then affecting IgM^+ B cell functionality. It has to be taken into consideration that although human B cells have been shown to directly react to β-glucans after recognition through dectin-1 [29], whether rainbow trout B cells express this receptor or any of the others known to interact with β-glucans has not yet been revealed in this nor in any other fish species.

One of the first things that we observed by flow cytometry was that β-glucans increased the size of IgM^+ B cells in the cultures. It is well known that upon antigen encounter, B cells increase their size as they differentiate towards IgM-secreting cells, plasmablasts and eventually terminally differentiated plasma cells [36,37,39]. Interestingly, this effect was observed both in IgM^+IgD^+ and IgM^+IgD^- B cell populations, despite the fact that IgM^+IgD^- B cells have been identified as cells that have already initiated this differentiation process [36,37,39], suggesting that these cells can experience a further differentiation in response to β-glucans. Similarly, IgM^+IgD^- B cells in rainbow trout gills and skin further increased their size in response to infection with *Yersinia ruckeri* [40].

We also observed that the presence of β-glucans significantly increased the number of IgM-secreting cells in the cultures, hence confirming that these molecules mediate the differentiation of IgM^+ B cells. These effects were exerted by β-glucans alone and also when combined with another stimulus, TNP-LPS, a good inducer of fish B cell differentiation [30]. The fact that β-glucans promoted the differentiation of IgM^+ B cells in rainbow trout was further confirmed in an *in vivo* trial, in which fish were fed a control diet or a diet supplemented with β-glucans for one month. We observed that the number of IgM-secreting cells in blood leukocyte cultures obtained from fish fed the β-glucan-enriched diet was significantly higher than that of fish fed the control diet. In

correlation with this, the percentage of cells that had lost surface IgD expression ($IgM^{+}IgD^{-}$ B cells) was also higher in these fish when compared to fish fed the control diet.

Nonetheless, the effects exerted on IgM^{+} B cells by β-glucans were not always stimulatory. Our *in vitro* studies revealed that the presence of β-glucans in the cultures provoked a decrease in surface MHC II levels and antigen-processing capacities. While MHC II significantly decreased in response to β-glucans in both non-stimulated and TNP-LPS-stimulated cultures, in the case of the antigen processing capacities, this reduction was only significantly detected in stimulated cultures. It is well known in mammals that as B cells differentiate, they typically reduce the machinery related to antigen presentation, including the expression of MHC II and their antigen processing capacities [41]. In this sense, these effects of β-glucans could be interpreted as the consequence of B cells differentiating to plasmablasts/plasma cells. However, previous studies have shown that in fish, in many occasions, B cells differentiate into IgM secreting cells but still maintain their antigen processing and presenting capacities [37,42]. Similarly, mammalian plasma cells differentiated in response to TI antigens have been seen to maintain elevated MHC II surface expression [43]. Therefore, it seems that this reduction of the antigen-presenting machinery is a peculiar characteristic of the response of rainbow trout B cells to β-glucans, and not a general effect. Interestingly, although one would expect that cells with an increased IgM secretory capacity would also have a decreased phagocytic capacity, the phagocytic capacity of IgM^{+} B cells was also increased in response to β-glucans. How β-glucans are capable of exerting these two effects is something that should be further investigated in the future.

Additionally, we analyzed the capacity of β-glucans to promote the proliferation of blood IgM^{+} B cells. In this case, the two highest doses of β-glucans on their own were capable of inducing the proliferation of a low but significant number of IgM^{+} B cells. However, when the proliferation of B cells was induced with TNP-LPS, the presence of β-glucans in the cultures significantly reduced this proliferative activity. Whether this effect is a consequence of some IgM^{+} B cells differentiating to plasmablasts/plasma cells and losing their ability to further respond to TNP-LPS is still only a hypothesis that should be further confirmed.

The results presented in this study significantly increase our knowledge on how β-glucans affect IgM^{+} B cells, something only scarcely studied to date. Interestingly, the effects observed in rainbow trout B cells are quite different to those previously reported in human B cells, pointing to specific effects on different species, probably as a result of a species-specific pattern of expression of receptors that can sense β-glucans. Hence, β-glucans have no proliferative effects on human B cells, nor they increase IgM secretion [29]. In contrast, they induce the production of pro-inflammatory cytokines by B cells [29]. In rainbow trout, we have demonstrated that β-glucans promote the differentiation of B cells to IgM-secreting cells, as established both in *in vivo* and *in vitro* trials. Furthermore, β-glucans slightly induced the proliferation of IgM^{+} B cells on their own but reduced that induced by TNP-LPS. Negative effects concerning the antigen-presenting capacities of IgM^{+} B cells were also observed, as β-glucans reduced the MHC II surface expression and antigen processing capacities, especially upon stimulation with TNP-LPS. However, positive effects on the phagocytic capacity of these cells were observed. The information provided in this work is valuable to better understand the effects that β-glucans may provoke on adaptive immune responses in teleost fish and will be of value to define their potential as vaccine adjuvants.

CRediT authorship contribution statement

D. Martín: Data curation, Formal analysis, Methodology. **M.C. Ordás:** Data curation, Formal analysis. **E. Morel:** Data curation, Formal analysis, Methodology, Writing – review & editing. **N. Nuñez-Ortiz:** Methodology, Writing – original draft. **P. Díaz-Rosales:** Methodology, Writing – review & editing. **Samuel Vicente-Gil:** Data curation, Methodology. **C. Zarza:** Conceptualization, Writing – review & editing. **L. Jensen:** Conceptualization, Writing – review & editing. **C. Tafalla:** Conceptualization, Supervision, Writing – original draft.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2024.109740.

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