Developmental and Comparative Immunology 111 (2020) 103752

Contents lists available at ScienceDirect

Developmental and Comparative Immunology

journal homepage: www.elsevier.com/locate/devcompimm

Perspective

Distinct modes of action of CD40L and adaptive cytokines IL-2, IL-4/13, IL-10 and IL-21 on rainbow trout IgM$^+$ B cells

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ARTICLE INFO

Keywords:
B cells
CD40L
IL-2B
IL-4/13
IL-10
IL-21

ABSTRACT

In mammals, conventional B (B2) cells are activated within lymphoid follicles through a close relationship with T follicular helper (Tfh) cells. The interaction between CD40 expressed on B cells and its ligand (CD40L) expressed on Tfh cells is a key signal that regulates the formation of germinal centers (GCs), B cell survival, proliferation and differentiation to plasma cells (PCs) or memory cells. Additionally, certain soluble cytokines produced by T cells also strongly condition the outcome of this interaction. Despite the many differences found between fish B cells and mammalian B2 cells, and the lack of conventional GCs, rainbow trout IgM$^+$ B cells have been shown to be stimulated by CD40L, however, whether cytokines commonly produced by T cells can further modulate this response has never been addressed to date. Thus, in this study, we determined the effects of recombinant rainbow trout adaptive cytokines interleukin 2B (IL-2B), IL-4/13A, IL-4/13B, IL-10 and IL-21 (cytokines known to activate B cells in mammals) on splenic IgM$^+$ B cells alone or in combination with CD40L. We studied how these cytokines and CD40L cooperated to promote IgM$^+$ B cell survival, proliferation and IgM secretion. The results obtained provide valuable information for the first time in teleost fish on how different T cell signals cooperate to activate B cells in the absence of GCs.

1. Introduction

In mammals, thymus-dependent (TD) antigens activate conventional B (B2) cell immune responses in the lymphoid follicles inducing the generation of germinal centers (GCs). Within these sites, B cells divide and differentiate into antibody-secreting cells (ASCs) that eventually reach a terminal state as plasma cells (PC), or into memory B cells (MacLennan, 1994). These responses involve the interaction of B2 cells with T follicular helper (Tfh) cells promoting proliferation, isotype switching from IgM to more specialized Igs (IgA, IgE and IgG) and antibody secretion (Crotty, 2011; Ma et al., 2012). Collaboration between B and T cells starts with the presentation of the antigen through MHC-class II molecules expressed by B cells (Chesnut and Grey, 1981; Lanzavecchia, 1985). Among the signals involved in cooperation between B2 cells and Tfh cells, the interaction between CD40 expressed on B cells and its ligand (CD40L) transitionally expressed on activated Tfh cells, is critical, stimulating the formation of GCs and promoting B cell survival, proliferation and differentiation (Noelle, 1996). This CD40$^-$CD40L ligation also up-regulates IRF4 and triggers NF-κB translocation within the B cell. IRF4 then down-regulates Bcl6, a repressor of STAT3, IRF4 and Blimp1 which are important factors for PC development (Ding et al., 2013). However, as part of this interaction, certain soluble cytokines produced by Tfh cells such as interleukin 2 (IL-2), IL-4, IL-10, IL-13 or IL-21 also strongly condition B cell responses within the GC (Hodgkin et al., 1990; Noelle et al., 1989; Rush and Hodgkin, 2001). In mammals, these cytokines, have been shown to synergize with CD40L, regulating multiple B cell functions such as lymphocyte proliferation (Armitage et al., 1995; Jelinek and Lipsky, 1985; Kishimoto, 1985), Ig secretion (Armitage et al., 1993; Maliszewski et al., 1993) or class switch recombination (CSR) (Arpin et al., 1995; Coffman et al., 1993; Stavnezer, 1996).

In teleost fish, only three Ig classes have been described to date, IgM, IgD and IgT. IgM and IgD are co-expressed on the surface of most naïve B cells. Upon antigen recognition, as described in mammals, these fish B cells have been shown to loose IgD surface expression (Granja and Tafalla, 2019). However, as also reported in specific human and mice mucosal compartments (Arpin et al., 1998; Chen et al., 2009; Koelsch et al., 2007), B cells exclusively expressing IgD on the cell surface have been identified in catfish (*Ictalurus punctatus*) blood (Edholm et al., 2010) or rainbow trout (*Oncorhynchus mykiss*) gills (Castro et al., 2014) and intestine (Perdiguero et al., 2019). IgT, on the other hand, is a teleost-specific Ig expressed on a distinct B cell lineage

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https://doi.org/10.1016/j.dci.2020.103752
Received 23 April 2020; Received in revised form 20 May 2020; Accepted 20 May 2020
Available online 22 May 2020

and IgT$^+$ B cells have been reported as specialized in mucosal responses since the ratio of IgT$^+$ B cells to IgM$^+$ B cells is higher in mucosal surfaces, and IgT responses seem to be restricted to mucosal compartments in response to some parasitic infections (Xu et al., 2016; Zhang et al., 2010). Thus, no CSR (replacement of IgM and IgD constant chains by other Ig chains that confer different effector functions such as IgA, IgE or IgG) has ever been reported in teleost fish. Furthermore, teleost fish do not have lymph nodes and do not form conventional GCs. All these differences suggest that teleost B cell responses could be equivalent to mammalian extrafollicular IgM responses. In line with this hypothesis, fish B cells have been shown to share many phenotypic and functional characteristics of mammalian B1 cells, innate B cells that immediately respond to antigens in the absence of T cell help to produce natural antibodies that block pathogens until a specific immune response is mounted (Baumgarth, 2011). For example, fish B cells, as mammalian B1 cells, have been shown to: have a high phagocytic capacity (Li et al., 2006); constitutively express numerous pattern recognition receptors (PRRs) (Abos et al., 2013, 2015), B1-specific markers (Abos et al., 2018; Castro et al., 2015) and high levels of surface IgM and low levels of IgD (Abos et al., 2018); have a long-life in cell culture; be larger and more complex than B2 cells; and be unable to proliferate in response to cross-linking of the B cell receptor (BCR) (Abos et al., 2018). Despite all the differences between mammalian B2 cells and fish B cells and the lack of GCs, rainbow trout CD40L has been shown to be capable of stimulating fish IgM$^+$ B cells, up-regulating IgM$^+$ B cell survival and proliferation, and promoting a differentiation to PC in a similar way to mammalian CD40L on B2 cells (Granja et al., 2019).

To date, only few studies have investigated the effect of cytokines on lymphocyte activation and differentiation in teleost fish. For example, recombinant rainbow trout IL-21 has been shown to increase the expression of T and B cell marker genes in head kidney leukocyte (HKL) cultures (Wang et al., 2011). Furthermore, recombinant rainbow trout IL-2 induced the proliferation of blood leukocytes *in vitro*, up-regulating the expression of T cell markers (CD4, CD8), results that suggested that this cytokine is a T cell growth factor in teleosts as in mammals (Wang et al., 2018). In carp, recombinant IL-10 was shown to promote the differentiation and proliferation of mature B cells in peripheral blood leukocyte (PBL) cultures, while it stimulated the secretion of antigen-specific IgM in HKLs from immunized fish (Piazzon et al., 2015). Finally, in several teleost species, two cytokines (IL-4/13A, IL-4/13B) have been identified that seem to have arisen from an ancestral IL-4/13 gene as a consequence of a teleost-specific whole genome duplication, with this ancestral gene thought to have diverged into IL-4 and IL-13 later in vertebrate evolution (Wang and Secombes, 2015). In rainbow trout, both of these isoforms, IL-4/13A and IL-4/13B, increased the number of IgM secreting B cells in splenocyte cultures, but had no effect on the proliferation of IgM$^+$ B cells (Wang et al., 2016).

In this context, in the current study, we have studied the effects of different cytokines known to have a B cell-stimulating role in mammals (IL-2, IL-4/13A, IL4/13B, IL-10 and IL-21) on different aspects of rainbow trout B cell functionality. We have tested the effects of the cytokines alone or in combination with CD40L on B cell survival, proliferation and IgM secretion. This study provides valuable information to understand how different T cell signals cooperate in fish to activate B cells in the absence of cognate GCs.

2. Materials and Methods

2.1. Fish

Rainbow trout (*Oncorhynchus mykiss*) of approximately 100 g were obtained from Centro de Agricultura El Molino (Madrid, Spain) and maintained at the animal facilities of the Animal Health Research Center (CISA-INIA, *Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria*) in a recirculating water system at 14 °C, with a 12:12-h light/dark photoperiod. A commercial diet (Skretting) was supplied to the fish twice a day. Trout were acclimatized to laboratory conditions for at least 2 weeks before any experimental procedure. All of the experiments described comply with the Guidelines of the European Union Council (2010/63/EU) for use of laboratory animals and have been approved by the INIA Ethics Committee (CEEA PROEX002/17).

2.2. Leukocyte isolation and stimulation

Fish were sacrificed by benzocaine (Sigma) overdose and spleens collected. Single cell suspensions were obtained using 100 μm nylon cell strainers (BD Biosciences) and Leibovitz medium (L-15, Invitrogen) supplemented with 100 IU/ml penicillin and 100 μg/ml streptomycin (P/S, Life Technologies), 5% fetal calf serum (FCS, Life Technologies) and 10 U/ml heparin (Sigma). Cell suspensions were placed onto 30/51% discontinuous Percoll (GE Healthcare) density gradients and centrifuged at 500 × *g* for 30 min at 4 °C, without brake. Cells at the interface were collected and washed in L-15 containing P/S and 5% FCS. The viable cell concentration was determined by trypan blue (Sigma-Aldrich) exclusion.

Recombinant CD40L (GenBank Accession number NP_001118138) and cytokines IL-2B (GenBank Accession number HE805273), IL-4/13A (GenBank Accession number FN820501), IL-4/13B (GenBank Accession number HG794522), IL-10 (GenBank Accession number NP_001233279.1), IL-21 (GenBank Accession number FM883702) were produced in *Escherichia coli* as described before (Wang et al., 2011, 2016, 2018). For splenocyte stimulation, the recombinant interleukins were used at a final concentration of 200 ng/ml as this was the optimal concentration established in previous studies (Wang et al., 2011, 2016, 2018). Similarly, the optimal CD40L dose for B cell stimulation had been established to be 5 μg/ml (Granja et al., 2019). In all cases, the cytokines were evaluated alone or in combination with CD40L, while in some experiments IL-2B and IL-10 were also assessed together. Non-stimulated controls were always included. Wells treated with the same amount of protein buffer as that added to cytokine-treated wells were also included in initial experiments to verify the absence of effects on B cells.

2.3. Flow cytometry analysis

Rainbow trout splenocytes in L-15 medium containing P/S and 5% FCS were seeded in 24-well plates (Nunc) at a concentration of 2 × 10^6 cells/ml, stimulated as described above and cultured at 20 °C for 3 days. After this time, 4 × 10^5 splenocytes were collected, washed in staining buffer (phenol red-free L-15 medium supplemented with 2% FCS) and incubated with an anti-trout IgM [1.14 mAb mouse IgG1 coupled to R-phycoerythrin (R-PE), 1 μg/ml] in staining buffer for 30 min at 4 °C in the dark. Afterwards, cells were washed twice and the percentage of live IgM$^+$ B cells in the cultures analyzed on a FACS Celesta flow cytometer (BD Biosciences) equipped with BD FACSDiva™ software. Dead cells were excluded from the analysis after staining with 4′,6-diamine-2′-phenylindole dihydrochloride (DAPI, 0.2 μg/ml). Flow cytometry analysis was performed with FlowJo® v.10 (FlowJo LLC, Tree Star) following the gating strategy described in Fig. S1.

2.4. B cell proliferation

The Click-iT Plus EdU Flow Cytometry Assay Kit (Sigma) was used to quantify cell proliferation following the manufacturer's instructions. Spleen leukocytes were seeded at a concentration of 2 × 10^6 cells/ml in 96-well plates and incubated with the different stimuli for 3 days at 20 °C. Subsequently, 5-ethynyl-2′-deoxyuridine (EdU) was added to the cultures at a final concentration of 1 μM and the cells were incubated for an additional 24 h. After that time, treated and untreated cells were collected and stained with anti-IgM (1.14) conjugated to

allophycocyanin (1 μg/ml) for 30 min at 4 °C. The incorporation of EdU to the DNA was determined following the manufacturer's instructions and then analyzed by flow cytometry in a FACS Celesta flow cytometer. Flow cytometry analysis was performed with FlowJo® v.10.

2.5. ELISPOT

ELISPOT was used to measure the number of IgM-secreting B cells in spleen leukocytes. For this, splenocytes were added to 96-well plates in triplicate at a concentration of 5×10^4 cells per well. Cells were then stimulated with the indicated cytokines and/or CD40L for 48 h at 20 °C. During this time, ELISPOT plates containing Immobilon-P membranes (Millipore) were activated with 70% ethanol and coated with 2 μg/ml of an anti-trout IgM mAb (clone 4C10) overnight at 4 °C with shaking. Plates were then washed with sterile PBS 5 times and non-specific binding sites were blocked by incubation with 2% BSA in PBS for 2 h at room temperature (RT). After the 48 h incubation period, cells were transferred to ELISPOT plates and incubated for a further 24 h. Next, splenocytes were removed from the plates and plates were washed 5 times with PBS and blocked with 2% BSA in PBS for 1 h at RT. After blocking, biotinylated anti-trout IgM mAb (clone 4C10) was added to the plates (1 μg/ml) and incubated for 1 h at RT. Following additional washing steps (5 times in PBS), the plates were developed using streptavidin-HRP (Thermo Fisher Scientific) at 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, the number of spots in each well was determined using an AID iSpot Reader System (Autoimmun Diagnostika GMBH).

2.6. Statistical analysis

Data was analyzed using Microsoft Office Excel 2010. Statistical analyses were performed using a two-tailed Student's *t*-test with Welch's correction when the F test indicated that the variances of both groups differed significantly. The differences between the mean values were considered significant when $P \leq 0.05$.

3. Results

3.1. Effects of CD40L and IL-21 on the activation of rainbow trout IgM$^+$ B cells

In humans, IL-21 is considered the most potent inducer of terminal B cell differentiation. Tfh cells are the most abundant producers of IL-21 but also other T cells subsets can do so, including Th1 and Th17 (Chtanova et al., 2004; Rasheed et al., 2006). In rainbow trout, we found that IL-21, on its own, significantly increased the percentage of IgM$^+$ B cells (Fig. 1A) as well as the total number of IgM$^+$ B cells in cultures (Fig. S2). Additionally, it induced the proliferation of IgM$^+$ B cells (Fig. 1B) and also up-regulated the number of IgM-secreting cells in splenocyte cultures (Fig. 1C). As previously reported (Granja et al., 2019), CD40L up-regulated the number of IgM$^+$ B cells in the cultures (Fig. 1A; Fig. S2), induced their proliferation (Fig. 1B) and promoted IgM secretion (Fig. 1C). To study whether synergistic effects were found between CD40L and IL-21, we stimulated splenocytes with the combination of both stimuli, simultaneously added. Our results show that the combination of CD40L and IL-21 increased IgM$^+$ B cell numbers (Fig. 1A; Fig. S2), IgM$^+$ B cell proliferation (Fig. 1B) and IgM secretion (Fig. 1C) reaching values significantly higher than those obtained in response to either CD40L or IL-21 alone. The fold induction of EDU$^+$/IgM$^+$ cells in CD40L and IL-21 stimulated samples (14.5%) was bigger than the sum of the samples stimulated by CD40L (3.9%), and IL-21 (4.1%) alone, suggesting a synergistic effect on proliferation.

Interestingly, IL-21 alone strongly increased the proliferation of non-IgM cells in splenocyte cultures (Fig. 1B). Although these non-IgM proliferating cells could be IgT$^+$ B cells, it might also be possible that, as described in mammals, rainbow trout IL-21 promotes the proliferation of T cells. These effects were not further up-regulated when CD40L was present (Fig. 1B).

3.2. Effects of CD40L and IL-2 on the activation of rainbow trout IgM$^+$ B cells

In mammals, IL-2 is mainly secreted by Th1 cells activated through the TCR or by mitogens (Morgan et al., 1976; Smith, 1980, 1988). However, IL-2 has also been shown to be produced by murine DCs activated by Gram-negative bacteria and even by B cells under some circumstances (Gaffen et al., 1996; Granucci et al., 2001; Walker et al., 1988). Two different paralogs of IL-2 (IL-2A and IL-2B) have been identified in salmonids generated from the additional genome-wide duplication event that salmonids underwent through evolution. Both isoforms have been shown to up-regulate crucial Th1 and Th2 cytokines, chemokine and cytokine receptors and increase the phagocytic activity of blood myeloid cells (Wang et al., 2018). However, as IL-2B has been shown to promote a higher production of IFN-γ, sustain higher CD8 expression levels and induce PBL proliferation (Wang et al., 2018), this was the isoform used in our experiments.

Our results revealed that IL-2B alone significantly increased the number of IgM$^+$ B cells (Fig. 2A; Fig. S2), their proliferation (Fig. 2B) and up-regulated the number of IgM-secreting cells in cultures (Fig. 2C). When IL-2B was combined with CD40L the number of IgM$^+$ B cells in the cultures (Fig. 2A; Fig. S2) and the levels of IgM$^+$ B cell proliferation (Fig. 2B) were significantly higher than those observed in response to IL-2B or CD40L alone, suggesting an additive effect. This effect, however, was not observed when the number of IgM-secreting cells (Fig. 2C) was assayed, as in this case the levels reached by the combination of IL-2B and CD40L were not significantly higher than those obtained in response to either stimulant independently.

As occurred with IL-21, IL-2B significantly increased the proliferation of IgM$^-$ cells in splenocyte cultures (Fig. 2B). Although these cells could also be IgT$^+$ B cells, our results could indicate that, as with mammalian IL-2, rainbow trout IL-2 promotes the proliferation of T cells. These effects were not further up-regulated when CD40L was present (Fig. 2B).

3.3. Effects of CD40L and IL-10 on the activation of rainbow trout IgM$^+$ B cells

IL-10, mainly produced by CD4$^+$ T cells and monocytes/macrophages, is a strong stimulator of Ig secretion by activated B cells in mammals (Wolk et al., 2002). When assessing the effects of IL-10 on rainbow trout B cells, we observed that IL-10 on its own significantly increased the number of IgM$^+$ B cells (Fig. 3A; Fig. S2) and the number of IgM-secreting cells (Fig. 3C) in splenocyte cultures. However, this cytokine was not able to induce IgM$^+$ B cell proliferation by itself (Fig. 3B), suggesting it is a survival factor of IgM$^+$ B cells. In contrast, IL-10 in combination with CD40L strongly induced the proliferation of IgM$^+$ B cells, reaching values significantly higher than those observed in cultures treated by CD40L alone (Fig. 3B). Interestingly, the combination of CD40L and IL-10 also significantly induced the proliferation of IgM$^-$ cells in the cultures, despite the fact that neither CD40L nor IL-10 alone were able to stimulate the proliferation of these cells (Fig. 3B). Regarding IgM$^+$ B cell survival and IgM secretion, additive effects of CD40L and IL-10 were also observed. Thus the levels of IgM$^+$ B cell survival (Fig. 3A) and the number of IgM-secreting cells in cultures (Fig. 3C) reached in response to both stimuli combined were significantly higher than those observed in splenocytes treated with IL-10 or CD40L alone.

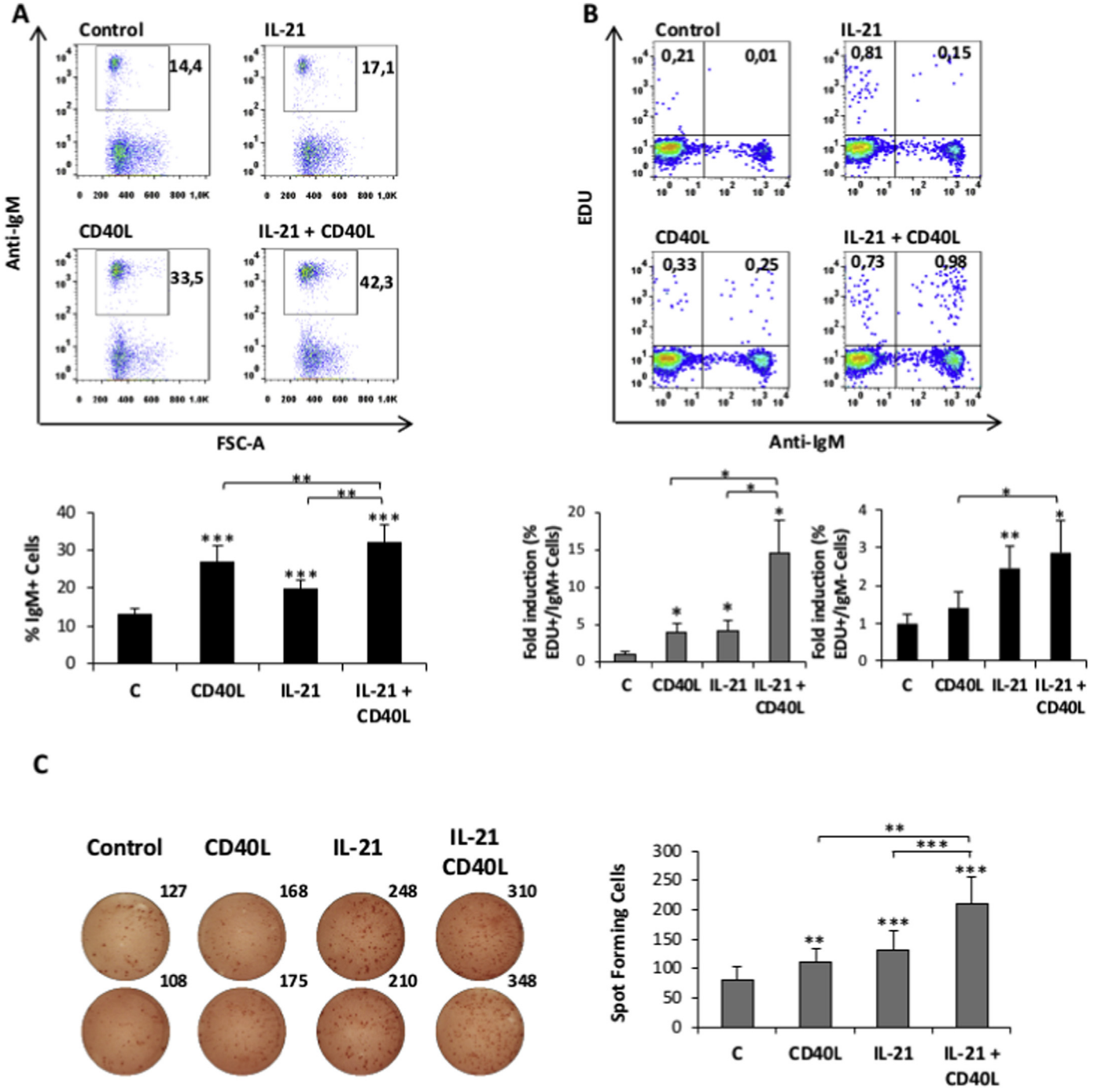

Fig. 1. Effects of CD40L and IL-21 on rainbow trout B cells. Splenocytes were incubated with CD40L (5 μg/ml), IL-21 (200 ng/ml) or a combination of both for 3 days at 20 °C. Non-stimulated controls were also included. (A) Thereafter, the survival of IgM^+ B cells was evaluated by flow cytometry. Representative dot plots are shown along with a graph showing the percentages of IgM^+ cells (mean + SEM; n = 12 independent fish). (B) The percentage of proliferating IgM^+ B cells (EDU^+/IgM^+) and IgM^- cells (EDU^+/IgM^-) cells was determined after 3 days in culture followed by 24 h of incubation with EDU as described in Materials and Methods. Representative dot plots and percentage of EDU^+/IgM^+ B cells and EDU^+/IgM^- cells relative to control are represented (mean + SEM; n = 8 independent fish). (C) ELISPOT analysis of IgM-secreting cells. Duplicate wells from a representative experiment are shown along with the mean number of spot forming cells (mean + SEM; n = 12 independent fish). Asterisks denote significantly different values among groups as indicated (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

3.4. Effects of CD40L and IL-2B and IL-10 on the activation of rainbow trout IgM^+ B cells

In humans, IL-2 and IL-10 further synergize to promote proliferation of B cells and the secretion of substantial quantities of IgM, IgG and IgA. These effects are mediated by the induction of high affinity IL-2 receptors (IL-2Ra chain) on B cells by IL-10 (Fluckiger et al., 1993). To establish if this phenomenon also takes place in fish, we evaluated the effects of both IL-2B and IL-10 on IgM^+ B cells in the presence of CD40L. Our results showed that when both IL-10 and IL-2B were present, in addition to CD40L, the number of IgM^+ B cells was significantly higher than that observed in response to the combination of CD40L and IL-2B or to that of CD40L and IL-10 (Fig. 4A; Fig. S2). The same pattern was observed when the number of IgM-secreting cells was analyzed in the cultures (Fig. 4C). These effects of IL-2B and IL-10 on CD40L-activated B cells were not observed in the proliferation studies, as in this

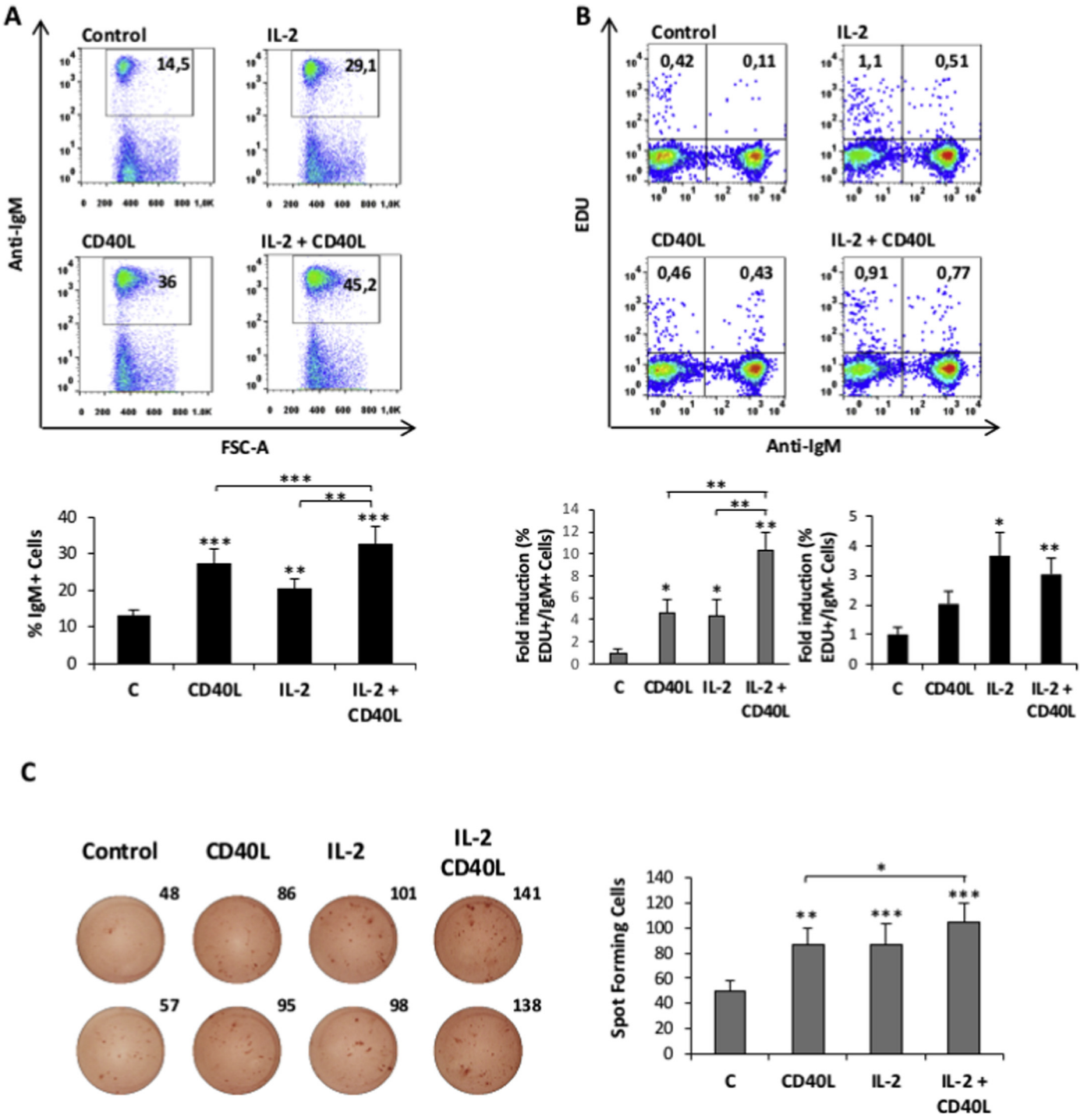

Fig. 2. Effects of CD40L and IL-2 on rainbow trout B cells. Splenocytes were incubated with CD40L (5 μg/ml), IL-2b (200 ng/ml) or a combination of both for 3 days at 20 °C. Non-stimulated controls were also included. (A) Thereafter, the survival of IgM^+ B cells was evaluated by flow cytometry. Representative dot plots are shown along with a graph showing the percentages of IgM^+ cells (mean + SEM; n = 12 independent fish). (B) The percentage of proliferating IgM^+ B cells (EDU^+/IgM^+) and IgM^- cells (EDU^+/IgM^-) cells was determined after 3 days in culture followed by 24 h of incubation with EDU as described in Materials and Methods. Representative dot plots and percentage of EDU^+/IgM^+ B cells and EDU^+/IgM^- cells relative to control are represented (mean + SEM; n = 5 independent fish). (C) ELISPOT analysis of IgM-secreting cells. Duplicate wells from a representative experiment are shown along with the mean number of spot forming cells (mean + SEM; n = 12 independent fish). Asterisks denote significantly different values among groups as indicated (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

case the number of proliferating IgM^+ B cells in response to IL-10, IL-2B and CD40L was not significantly higher than that reached in response to CD40L and IL-10 (Fig. 4B).

3.5. CD40L and IL-4/13 do not exert additive effects on rainbow trout IgM^+ B cells

IL-4 is a crucial cytokine in the development of Th2 immune responses in mammals. It is mainly produced by T cells, mast cells and basophils; and is known to regulate several functions of B cells

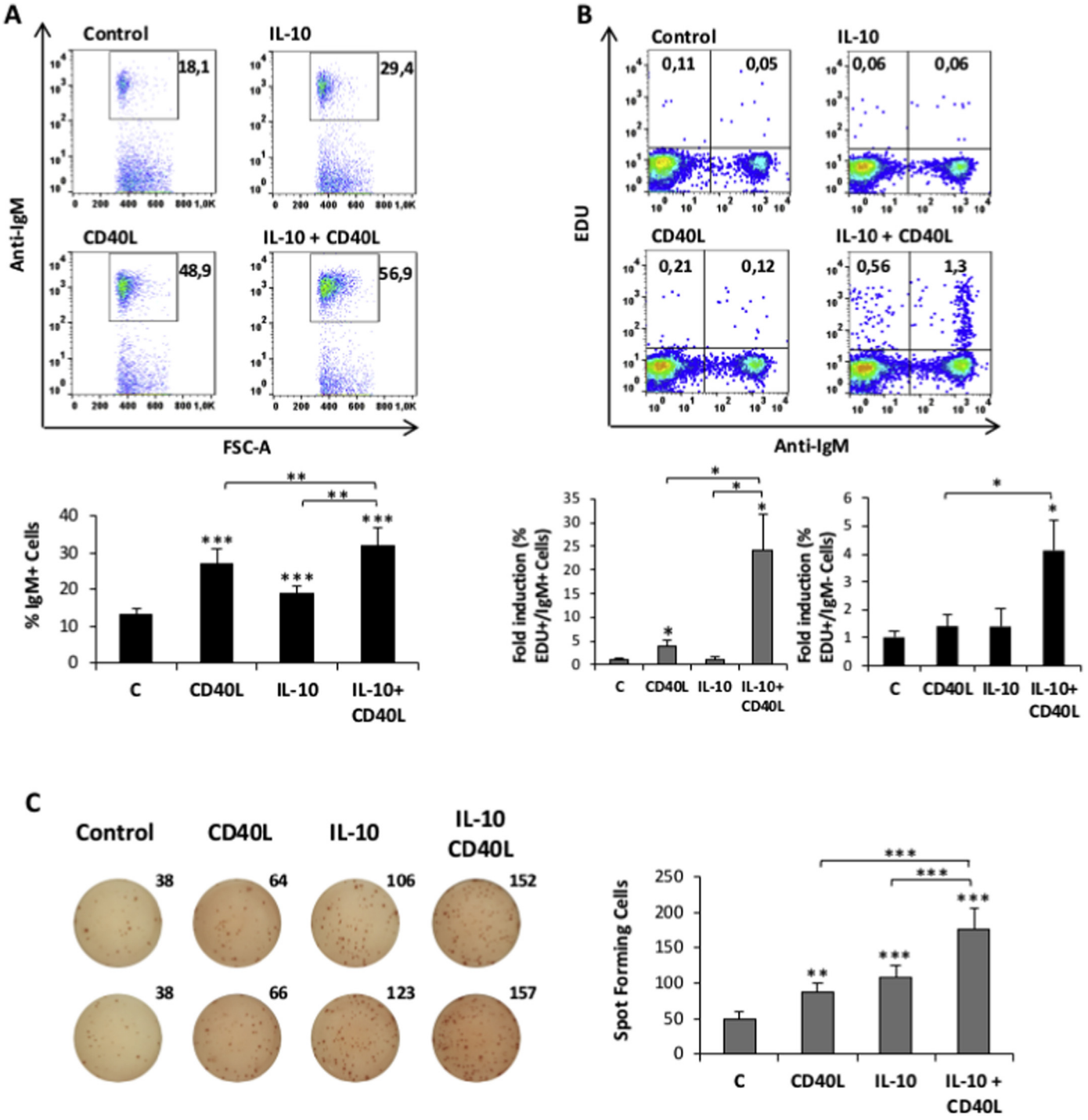

Fig. 3. Effects of CD40L and IL-10 on rainbow trout B cells. Splenocytes were incubated with CD40L (5 μg/ml), IL-10 (200 ng/ml) or a combination of both for 3 days at 20 °C. Non-stimulated controls were also included. (A) Thereafter, the survival of IgM^+ B cells was evaluated by flow cytometry. Representative dot plots are shown along with a graph showing the percentages of IgM^+ cells (mean + SEM; n = 12 independent fish). (B) The percentage of proliferating IgM^+ B cells (EDU^+/IgM^+) and IgM^- cells (EDU^+/IgM^-) cells was determined after 3 days in culture followed by 24 h of incubation with EDU as described in Materials and Methods. Representative dot plots and percentage of EDU^+/IgM^+ B cells and EDU^+/IgM^- cells relative to control are represented (mean + SEM; n = 8 independent fish). (C) ELISPOT analysis of IgM-secreting cells. Duplicate wells from a representative experiment are shown along with the mean number of spot forming cells (mean + SEM; n = 12 independent fish). Asterisks denote significantly different values among groups as indicated (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

(Banchereau et al., 1993; Finkelman and Urban, 2001; Mosmann et al., 1986). As previously reported (Wang et al., 2016), neither IL-4/13A or IL-4/13B on their own were able to up-regulate IgM^+ B cell survival or proliferation (Fig. 5A and B). In contrast, a low but significant enhancement in the number of IgM-secreting cells was observed in cultures stimulated with either IL-4/13A or IL-4/13B (Fig. 5C). When we assessed the effects of IL-4/13A and B in combination with CD40L on IgM^+ B cell survival, no additive/synergistic effects were observed (Fig. 5A). In fact the levels of IgM^+ B cell survival observed in response to either IL-4/13A and CD40L or IL-4/13B and CD40L were significantly lower than those observed in cultures treated with CD40L alone (Fig. 5A; Fig. S2). Additionally, no significant differences were

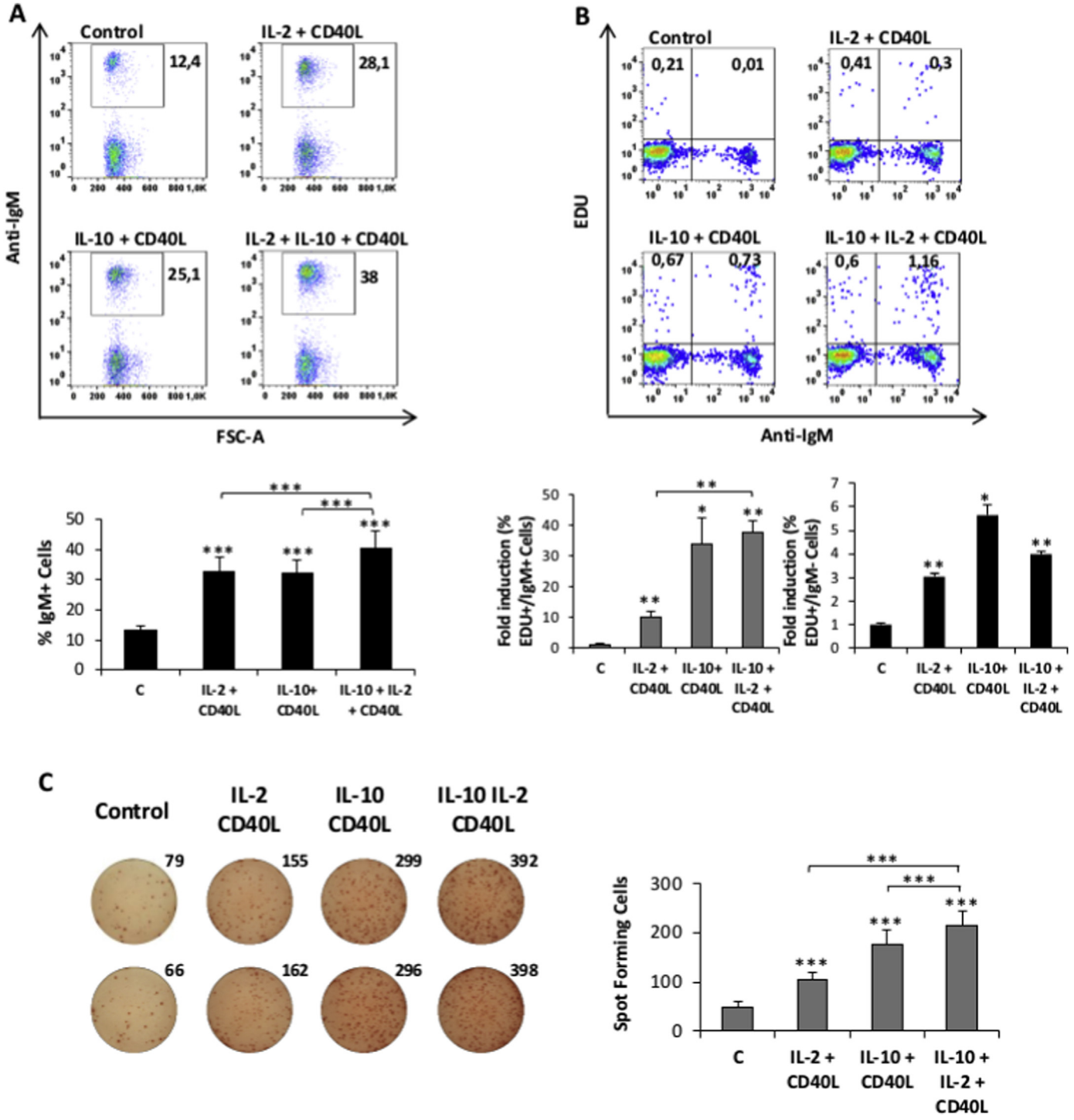

Fig. 4. Effects of CD40L in combination with both IL-2 and IL-10 on rainbow trout B cells. Splenocytes were incubated with CD40L (5 μg/ml), CD40L in combination with IL-2b (200 ng/ml), CD40L in combination with IL-10 (200 ng/ml) or the two cytokines together with CD40L for 3 days at 20 °C. Non-stimulated controls were also included. (A) Thereafter, the survival of IgM^+ B cells was evaluated by flow cytometry. Representative dot plots are shown along with a graph showing the percentages of IgM^+ cells (mean + SEM; n = 12 independent fish). (B) The percentage of proliferating IgM^+ B cells (EDU^+/IgM^+) and IgM^- cells (EDU^+/IgM^-) cells was determined after 3 days in culture followed by 24 h of incubation with EDU as described in Materials and Methods. Representative dot plots and percentage of EDU^+/IgM^+ B cells and EDU^+/IgM^- cells relative to control are represented (mean + SEM; n = 5 independent fish). (C) ELISPOT analysis of IgM-secreting cells. Duplicate wells from a representative experiment are shown along with the mean number of spot forming cells (mean + SEM; n = 12 independent fish). Asterisks denote significantly different values among groups as indicated (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

observed in the percentage of proliferating IgM^+ B cells between cultures stimulated with CD40L alone and those stimulated with CD40L combined with IL-4/13A or IL-4/13B (Fig. 5B). Regarding the amount of IgM-secreting cells in cultures, CD40L combined with IL-4/13A or IL-4/13B provoked effects that were not significantly higher than those induced by the cytokines alone (Fig. 5C). In conclusion, no additive/synergistic effects of IL-4/13A or IL-4/13B and CD40L were observed on rainbow trout IgM^+ B cells.

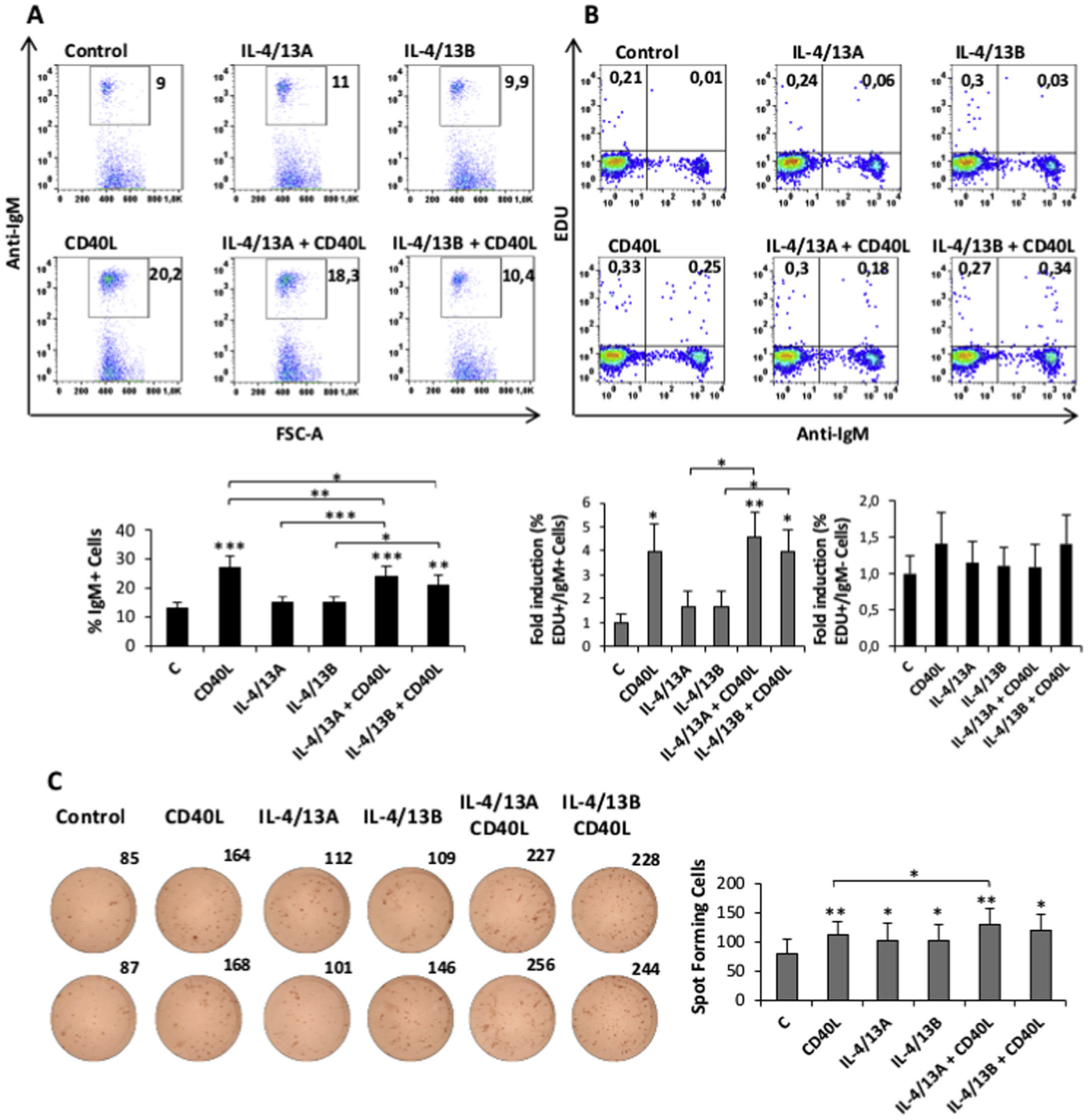

Fig. 5. Effects of CD40L and IL-4/13 on rainbow trout B cells. Splenocytes were incubated with CD40L (5 μg/ml), IL-4/13A (200 ng/ml), IL-4/13B (200 ng/ml), CD40L in combination with IL-4/13A, or CD40L in combination with IL-4/13B for 3 days at 20 °C. Non-stimulated controls were also included. (A) Thereafter, the survival of IgM^+ B cells was evaluated by flow cytometry. Representative dot plots are shown along with a graph showing the percentages of IgM^+ cells (mean + SEM; n = 12 independent fish). (B) The percentage of proliferating IgM^+ B cells (EDU^+/IgM^+) and IgM^- cells (EDU^+/IgM^-) cells was determined after 3 days in culture followed by 24 h of incubation with EDU as described in Materials and Methods. Representative dot plots and percentage of EDU^+/IgM^+ B cells and EDU^+/IgM^- cells relative to control are represented (mean + SEM; n = 8 independent fish). (C) ELISPOT analysis of IgM-secreting cells. Duplicate wells from a representative experiment are shown along with the mean number of spot forming cells (mean + SEM; n = 12 independent fish). Asterisks denote significantly different values among groups as indicated (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

4. Discussion

Despite the lack of CSR and follicular structures (Zapata et al., 1995), the limited Ig isotype repertoire (Cain et al., 2002; Warr, 1995) and the resemblance of fish B cells to mammalian B1 cells in many functional and phenotypic aspects (Abos et al., 2013, 2015, 2018; Castro et al., 2015; Li et al., 2006; Scapigliati et al., 2018; Zhang et al., 2017), fish B cells are capable of responding to CD40L (Granja et al., 2019). However, the lack of organized lymphoid structures where B and T cells can interact in a concerted fashion will surely influence how fish

B cells respond to different types of antigens and T cell-derived signals. For example, this could be the reason why rainbow trout B cells, in the absence of adjuvants, are only weakly activated by TD antigens such as 2,4,6-trinitrophenyl-keyhole limpet hemocyanin (TNP-KLH), while they are strongly activated by thymus-independent (TI) antigens that substitute T cell help by stimulation of innate receptors (Granja et al., 2019). Interestingly, in rainbow trout, CD40L was shown to synergize with TNP-LPS (a TI antigen) but not with TNP-KLH (Granja et al., 2019). Furthermore, accumulating evidence in mammals (Clark, 1997; Ma and Clark, 2009) and fish (Granja et al., 2019), also point to the fact that CD40L can be provided by other cell types rather than only T cells, such as for example, DCs. Thus, it now seems clear that there is not a clear dichotomy between TD and TI responses and there might be many alternative ways to activate B cells both in mammals and teleost fish in which both T cell-derived signals and innate signals cooperate to fully activate B cells. In this context, it seemed extremely relevant to determine if other cytokines commonly secreted by T cells during an adaptive immune response were influencing the response of fish B cells to CD40L in a similar way to that described for mammalian B cells. In our studies, we stimulated complete cultures with the different cytokine combinations and then studied the effects on IgM^+ B cells. Therefore, indirect effects exerted by the response of other cell types that then influence B cell functionality are possible. However, direct effects of the cytokines are presumed given the fact that rainbow trout splenic IgM^+ B cells constitutively express receptors for all these cytokines (Granja et al., 2019).

We first determined the effects of IL-21, considered the most potent inducer of terminal B cell differentiation in mammals (Crotty, 2011; Ma et al., 2012; Tangye et al., 2013). Rainbow trout IL-21, by itself, significantly increased IgM^+ B cell survival and proliferation and also up-regulated the number of IgM-secreting cells in splenocyte cultures, known to correlate with an increased IgM concentration in the culture media (data not shown). These results revealed a B cell-stimulating role for IL-21 for the first time in teleost fish. In contrast, in mammals, when no appropriate T cell help or specific TLR stimulation occurs, IL-21 induces B cell apoptosis (Konforte et al., 2009). All these effects provoked by rainbow trout IL-21 were significantly increased in the presence of CD40L, demonstrating a strong additive action between both stimuli. Mammalian IL-21 has also been shown to promote strong B cell proliferation following CD40 ligation on both naïve and memory B cells from PBLs and cord blood, while CD40 engagement alone results in minimal cell expansion (Ettinger et al., 2005; Parrish-Novak et al., 2000). Additionally, it has been established that CD40 ligation together with IL-21 induces CSR, Ig secretion and PC differentiation through activation-induced cytidine deaminase (AID) and Blimp-1 induction (Avery et al., 2010; Bryant et al., 2007; Diehl et al., 2008; Ettinger et al., 2005; Kuchen et al., 2007; Recher et al., 2011). Thus, splenic GC or blood B cells produce 20-fold more Ig-secreting cells after IL-21 and CD40L treatment than in response to CD40L combined with IL-10 (Ettinger et al., 2005). In our studies, although these differences were not as high, the number of IgM-secreting cells obtained in cultures treated with CD40L and IL-21 was also higher than that observed in cultures stimulated with CD40L and IL-10. Interestingly, previous studies from our group showed that two isoforms of IL-21 receptor were transcriptionally up-regulated in rainbow trout splenic B cells in response to a TI stimulant (Granja et al., 2019). Taking these results into consideration as well as the fact that IL-21 by itself activates B cells, it seems that rainbow trout IL-21 produced by T cells has the capacity to promote the activation/differentiation of B cells more broadly than in the context of TD responses.

As a well-known T cell growth factor, IL-2 has been shown to induce the expansion and survival of activated T cells, promoting naïve T cell differentiation into Th1 and Th2 cells (Liao et al., 2011, 2013). However, IL-2 also activates natural killer (NK) cells and Ig secretion by activated B cells (Gold and DeFranco, 1994; Yu et al., 2000). In these species, CD40L/CD40 engagement induces naïve B cells to become IL-2 responsive (by up-regulating the expression of the IL-2 receptor) driving them to proliferate and secrete IgM (Johnson et al., 1998). Thus, the presence of both stimuli is required to promote PC differentiation, inducing higher levels of IgM, IgG1 and IgA (Armitage et al., 1993). In contrast, in our studies, rainbow trout IL-2B significantly increased IgM^+ B cell survival and proliferation and the number of IgM-secreting cells in cultures, in the absence of CD40L. However, co-stimulation of splenocytes with CD40L and IL-2B promoted a significant increase in the percentage of surviving IgM^+ B cells and the levels of IgM^+ B cell proliferation in comparison to those reached in cells treated with CD40L or IL-2B alone. In contrast, no additive effects were observed regarding IgM secretion.

IL-10 is a multifaceted cytokine with inhibitory and stimulatory actions in the immune system (Fiorentino et al., 1989). It has been shown to play an important role in peripheral tolerance and in the inhibition of the immune responses through the induction of regulatory T and B cells (Wolk et al., 2002). However, IL-10 also prevents the apoptosis of B cells and enhances their proliferation, differentiation and the expression of MHC class II molecules (Go et al., 1990; Rousset et al., 1995). Regarding its interaction with CD40L, Rousset and colleagues found that when CD40 was engaged in purified B cells, IL-10 promoted the generation of higher levels of IgM, IgG and IgA than those induced by IL-2, IL-4 or the combination of IL-4 and IL-10 (Rousset et al., 1992). Our results revealed a significant increase in the number of IgM-secreting cells in splenocyte cultures after stimulation with IL-10 alone, an effect that was significantly enhanced in the presence of CD40L, showing a strong additive effect between the two stimuli. In agreement with the results obtained by Rousset and colleagues (Rousset et al., 1992), in rainbow trout, the number of IgM secreting cells in cultures stimulated with CD40L and IL-10 were also higher than those induced by the combination of CD40L and IL-2, IL-4/13A or IL-4/13B. Interestingly, in carp, IL-10 was shown to significantly increase IgM secretion only in HKLs obtained from immunized fish but not from naïve B cells, leading the authors to infer that BCR activation by specific antigen was required for B cells to secrete IgM in response to IL-10 (Piazzon et al., 2015). In our case, IL-10 by itself was able to induce a significant increase of Ig secretion in spleen leukocytes obtained from naïve fish. Nevertheless, it might be possible that the response to IL-10 would be even stronger in previously stimulated fish, as transcriptional studies performed by our group revealed that the mRNA levels of the IL-10 receptor were significantly up-regulated in rainbow trout splenic B cells in response to either TD or TI stimuli (Granja et al., 2019). However, these different responses could also be due to a disparity between the two species or a consequence of different organs and/or detection methods used in the two studies. Different responses between the effects of carp IL-10 and rainbow trout IL-10 on B cell proliferation might also be possible. Carp IL-10 was able to induce a significant IgM^+ B cell proliferation in naïve or immunized fish PBLs but not in HKLs (Piazzon et al., 2015). In rainbow trout splenocytes, however, IL-10 alone was not capable of inducing significant IgM^+ B cell proliferation rates. In contrast, a dramatic increase in the percentage of EDU^+/IgM^+ B cells was obtained in response to both CD40L and IL-10, reaching values approximately 24-fold higher than those of control cells, 20-fold higher than those reached by IL-10 alone and 6-fold higher than those achieved in response to CD40L. Similarly, a strong B cell proliferation has also been demonstrated in human anti-CD40 activated B cells stimulated with IL-10 (Rousset et al., 1991). The proliferation rates reached in response to CD40L and IL-10 were the highest rates reached in our study, revealing a stronger synergistic effect between CD40L and IL-10 than that with other cytokines. This strong synergistic effect on IgM^+ B cell proliferation was not increased further by addition of IL-2 even though the survival rates obtained when IL-2 was included in the cultures were significantly higher than those obtained in response to CD40L and IL-10 alone.

In rainbow trout, as reported before (Wang et al., 2016), both IL-4/13 isoforms increased the number of IgM-secreting B cells in cultures

but had no effects on IgM$^+$ B cell survival or proliferation. In contrast, IL-4/13A induced a significant increase in the number of B cells in PBLs when administered intraperitoneally to zebrafish (Zhu et al., 2012). In mammals, IL-4 has been shown to induce survival, proliferation and differentiation of B cells when continuous IL-4 and CD40 stimulation occurs (Banchereau et al., 1991; Rush and Hodgkin, 2001). However, in our studies, no additive effects were observed between CD40L and IL-4/13A or IL-4/13B suggesting that the way in which IL-4/13 affects B cells differs from the effects of mammalian IL-4 on B cells.

In conclusion, we have studied for the first time in teleost fish how cytokines commonly produced by Th cells regulate splenic B cells by themselves or in combination with CD40L. Our results demonstrate that rainbow trout IgM$^+$ B cells increase their survival in response to IL-2B, IL-10 and IL-21, while only IL-2B and IL-21 induced their proliferation. On the other hand, all cytokines tested (IL-2B, IL4/13A, IL4/13B, IL-10 and IL-21) up-regulated the number of IgM-secreting cells in splenocyte cultures. Regarding their additive effects, IL-10 and IL-21 cooperated with CD40L to further promote IgM$^+$ B cell survival, proliferation and IgM-secretion, whereas IL-2 cooperated with CD40L to up-regulate IgM$^+$ B cell survival and proliferation but not IgM secretion. In contrast, no additive effects were found between IL4/13A or IL4/13B and CD40L. Our results provide relevant information on how cytokines commonly produced by Th cells can modulate B cell functions in fish and how these signals cooperate with cognate T cell help to achieve a high level of B cell activation/differentiation. This information will contribute to a better understanding of how teleost B cells are influenced by T cell-derived signals in the absence of organized structures such as cognate GCs.

Declaration of competing interest

The authors declare no competing interests.

Acknowledgements

This work was supported by the European Research Council (ERC Consolidator Grant 725061) and by the Spanish Ministry of Science, Innovation and Universities (project AGL2017-85494-C2-1-R).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.dci.2020.103752.

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