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Short communication

Recombinant carp IL-4/13B stimulates *in vitro* proliferation of carp IgM$^+$ B cells

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ABSTRACT

Teleost IL-4/13B is a cytokine related to mammalian IL-4 and IL-13, of which hitherto the function had not been studied at the protein level. We identified an *IL-4/13B* gene in common carp (*Cyprinus carpio*) and expressed the recombinant protein (rcIL-4/13B). RcIL-4/13B was shown to stimulate proliferation of IgM$^+$ B cells, because after four days of stimulation the IgM$^+$ fraction of carp kidney and spleen leukocytes had formed many cell colonies, whereas such colonies were not found in the absence of rcIL-4/13B stimulation. After nine days of incubation with rcIL-4/13B these cells had proliferated to more than 3-to-7-fold higher numbers when compared to untreated cells. The proliferating cells contained a majority of IgM$^+$ cells but also other cells, as indicated by FACS and RT-PCR analyses. The important conclusion is that in fish not only IL-4/13A has B cell stimulating properties, as a previous publication has shown, but also IL-4/13B.

1. Introduction

In mammals, IL-4 and IL-13 are related cytokines that contribute to type 2 immunity [1]. Mammalian IL-4 was initially identified as a co-factor for inducing B cell growth, and even named "B cell growth factor" (BCGF) [7]. A similar B cell stimulating function was later also found for IL-13 [5]. IL-4 and IL-13 stimulate B cells to express IgE [5,6], which has an important role in anti-parasite immunity [3]. Teleost fish genes of the same family as mammalian *IL-4* and *IL-13* were designated *IL-4/13A* and *IL-4/13B*, because it can't be determined whether they are closer related to *IL-4* or to *IL-13* [10,13,17]. Teleost *IL-4/13A* and *IL-4/13B* map to two different chromosomes, and presumably originated from the whole genome duplication event early in the teleost line [13]. The functions of teleost IL-4/13 molecules are not well known yet, but gene expression studies indicate a divergent expression from the type 1 immunity marker molecule IFNγ, and suggest that the IL-4/13 molecules are involved in type 2 immunity like mammalian IL-4 and IL-13 [2,12,15,19]. Some differences in expression between teleost *IL-4/13A* and *IL-4/13B* were observed [9,19], and only in the promoters of *IL-4/13A* genes a well-conserved GATA-3 binding motif was identified [13]. Recombinant zebrafish IL-4/13A was found to stimulate zebrafish IgM$^+$ B cells [23], but, hitherto, functions of teleost IL-4/13B protein were not reported. In the present study we investigated whether carp IL-4/13B can stimulate IgM$^+$ B cell proliferation, and found such stimulatory effect indeed.

2. Materials and methods

2.1. Fish

Carp (*Cyprinus carpio*) weighting 40–60 g were purchased from a commercial farm and were kept at 25 °C in a recirculation system

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with filtered water and fed pelleted dry food once a day.

2.2. Molecular cloning of carp IL-4/13B

We determined carp *IL-4/13B* sequence (f; GenBank accession AB690830) from cDNA, starting our amplification strategy with primers derived from zebrafish *IL-4/13B* (we initiated these experiments before the carp genome sequence had been published), and by amplification and sequencing strategies (for some details see the Fig. S1 legend) similar to those described before [8]. *IL-4/13B* identity was confirmed by similarity with zebrafish *IL-4/13B*, and linkage with *KIF3A* gene as found in the carp genome scaffold sequence Contig8486 (Genbank accession LHQP01008480); for linkage of *IL4/13B* with *KIF3A* in zebrafish and other teleosts see Fig. S2 and [13].

2.3. Production and purification of recombinant protein of IL-4/13B

After addition of *Nde*I and *Bam*HI restriction motifs, the sequence encoding mature IL-4/13B (see Fig. S1) was cloned between the respective restriction sites of prokaryotic expression vector pET-16b (Novagen, USA), and by use of this construct N-terminal His-tagged IL-4/13B protein (rcIL-4/13B) was expressed in Rosetta-gami B (DE3) pLysS Competent cells (Novagen) and purified (similar as in Ref. [18]. For details see supplementary materials and methods.). The apparent molecular weight of rcIL-4/13B agreed well with its predicted molecular weight of 14.3 kDa (Fig. S3). Residual lipopolysaccharide (LPS) was shown to be less than 5 pg/mL according to a Limulus ES-II Single Test (Wako, Japan).

2.4. Isolation of carp surface-IgM$^+$cells

Surface-IgM$^+$ (sIgM$^+$) cells were isolated from carp kidney and spleen mononuclear leukocytes using magnetic activated cell sorting (MACS; Mini Macs, Miltenyi Biotec, Germany) similar, but not identical, to as done before [16]. In brief, kidney and spleen cells washed with minimal essential medium containing 2% fetal bovine serum (MEM-2) were applied to a Percoll density gradient of 1.080 g/mL and centrifuged at 400 × g for 30 min at 4 °C. To obtain sufficient numbers of sIgM$^+$ cells, it was necessary to use pooled leukocytes from head kidney and spleen of identical carp individuals. The cells at the interface were collected and washed twice with MEM-2, incubated with CI-5 hybridoma supernatant containing mouse anti-carp IgM monoclonal antibody (mAb) (for some details see supplementary materials and methods and Fig. S4) for 60 min at 4 °C, washed again two times with MEM-2, then incubated for 20 min at 4 °C with 100 μl of a 1:4 dilution of magnetic bead-conjugated goat anti-mouse Ig antibody (Miltenyi Biotec), and washed again two times with MEM-2. The cells were then resuspended in degassing MEM-2, and the cell suspension was applied to a MACS column for separation of sIgM$^+$ cells from sIgM$^-$ cells according to the manufacturer's protocol. RT-PCR and FACS analyses confirmed that the sIgM$^+$ cell fraction was effectively enriched for sIgM$^+$ B cells (Fig. 3 and Fig. S5).

2.5. Incubation of sIgM$^+$ cells with different concentrations of rcIL-4/13B

To assess the function of IL-4/13B in B cell proliferation, cells of the sIgM$^+$ fraction of carp kidney and spleen mononuclear leukocytes were incubated in the presence of different concentrations of rcIL-4/13B. Approximately 2 × 10^5 cells/well of MACS-separated sIgM$^+$ cells were cultured using E-RDF medium (Kyokuto, Japan) containing 20% fetal bovine serum and 2.5% carp pooled serum (E-RDF20/2.5) with 0, 100 or 1000 ng/mL of rcIL-4/13B in wells of a 48-well plate (Greiner bio one, Germany). Every few days these culture media were refreshed. Three independent experiments have been performed by using one fish for each experiment.

3. Results and discussions

After four days of incubation many colonies had formed in the presence of rcIL-4/13B, while colony formation was not observed in the absence of rcIL-4/13B (Fig. 1). Microscopy analysis of a glass smear of the cells showed that the majority of the cells were round cells with blue cytoplasm after Giemsa staining and a round-to-oval nucleus (Fig. 2A), and, as an indicator of active cell proliferation, mitotic figures were frequently observed (Fig. 2B). After nine days of incubation, when colonies fused with the neighboring colonies in fastest growing cells, the cells from all wells were collected and the total cell numbers counted. The numbers of cells which were incubated with rcIL-4/13B were 3-to-7-fold higher than those at the beginning of the experiment, while the numbers of cells incubated in the absence of rcIL-4/13B had increased less than 3-fold or had even decreased (Fig. 3). In two of the three examined individuals, the numbers of cells had increased in an rcIL-4/13B dose-dependent manner. FACS analysis revealed that the majority of these proliferating cells expressed IgM on their surface (black bars in Fig. 3), and RT-PCR analysis of transcripts for soluble and membrane IgM (cμ) confirmed the presence of IgM$^+$ cells among the proliferating cells (Fig. 4). Taken together, these results indicate that rcIL-4/13B has B cell stimulating properties. However, FACS analysis as well as RT-PCR analysis indicates that also non-B cell populations were present in our preparations, some of which were sensitive to rcIL-4/13B induced proliferation (Figs. 3 and 4 and Fig. S5). The type of protocol that we followed is always accompanied by some level of contamination, but some of the non-B cells might be isolated through means of their Fc receptors. Monocytes/macrophages and thrombocytes may have been labelled by binding of anti-IgM mAbs to Fc receptor bound carp IgM [30,31]. Despite this possible contamination, after nine days of incubation with rcIL-4/13B, the numbers of monocytes/macrophages and thrombocytes were suggested to be decreased since the expressions of their marker genes were reduced when compared to the expression in MACS-separated sIgM$^+$ cells ("Day 0" in Fig. 4). Freshly isolated MACS-separated sIgM$^+$ cells and rcIL-4/13B treated proliferating cells also expressed *IgZ* (*cζ*) (homologous to the *IgT* in salmonids; Fig. S5 and data not shown). Similar observations have been made in zebrafish, like carp belonging to the cyprinid fishes, where one of the zebrafish *IgZ* isotypes was also expressed in IgM$^+$ cells [24]. Likewise, in rainbow trout IgT$^+$ cells were reported to express *IgM* although its expression level was not high [21]. In our experiments, proliferating cells showed an enhanced expression of *TCRα*, suggesting a simultaneous increase in the number of T cells that were hardly found in the initial MACS-separated sIgM$^+$ cells (Fig. 4 and Fig. S5). We have previously shown that carp lymphocytes with T_H properties were preferentially selected by the applied cell culture conditions [19], while RT-PCR data in this study suggest that a T-cell proliferation is rather independent from the IL-4/13B concentration (Fig. 4). At least theoretically, our observed proliferation of carp B cells induced by rcIL-4/13B may not only have been caused by their direct interaction, but in addition or instead may have involved signaling cascades induced by stimulation of other cell types through rcIL-4/13B (e.g. see Ref. [25]). Future research should address whether rcIL-4/13B can also induce carp IgM$^+$ B cell proliferation in fully isolated conditions.

It is known that teleost fish B cells are difficult to culture for prolonged times, as shown in the present study for cells cultured without rcIL-4/13B. The only published exception is for catfish, a species in which various lymphocytes appear to have an