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NCCRP-1 Might Not be a Marker of so Called NCC Cells in Common Carp (*Cyprinus carpio*) Leukocytes

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Abstract: The purpose of this study was to verify whether NCCRP-1 is a marker of a unique type of cells in teleost, so-called NCC, or an ubiquitin, like in cytotoxic cells. Therefore, common carp peripheral blood leukocytes were isolated and tested for the binding of fluorescent NCCRP-1 antibody to stained MAIT, γδ T and T cells following stress treatments. The results were analyzed by a confocal microscope. The results revealed the presence of NCCRP-1 in γδ T, MAIT and T cells in more than one type of leukocytes. γδ T cells were the dominant population in carp leukocytes. Therefore, it was concluded that there might be no presence of NCC cells in the common carp leukocytes and that the NCC marker, NCCRP-1, acts probably as an ubiquitin in cytotoxic cells such as γδ T and MAIT cells, which were abundant in peripheral blood leukocytes of common carp.

Keywords: Gamma/Delta T Cell, MAIT Cell, NCCRP-1, Ubiquitin, NCC Cell

Introduction

In mammalian, cytotoxicity is due to a specific and unspecific strategy to eliminate pathogens and unwanted cells. The specific strategy involves $CD4^+$ and $CD8^+$ cytotoxic T lymphocytes (CTL), which requires antigen processing and presentation by Major Histocompatibility Complex (MHC) (Appay, 2004; Barry and Bleackley, 2002). The unspecific strategy involves mainly Natural Killer (NK) cells, which express activating and inhibitory receptors. NK-mediated cytotoxicity depends on the balance between these two receptor types (Vivier *et al*., 2011). To a lesser extent, γδ T (Holtmeier and Kabelitz, 2005), mucosal-associated invariant T (MAIT) (Le Bourhis *et al*., 2013; Napier *et al*., 2015; Gold and Lewinsohn, 2011; Eckle *et al*., 2015; Ussher *et al*., 2014) and natural killer T cells (NKT) (Bendelac *et al*., 2007; Matsuda *et al*., 2008; Rossjohn *et al*., 2012) cells also use unspecific strategy. Despite the antigen recognition difference, their mechanisms of killing are similar and involve the Ca^{2+}-dependent release of perforin and granzyme or receptor-mediated killing by Fas-Fas ligand interactions (Smyth *et al*., 2001; Trapani and Smyth, 2002).

Like mammals, teleosts also adopt a specific and nonspecific immune strategy in the cytotoxic response. So far, three types of cytotoxic cells have been identified in teleosts. These include nonspecific cytotoxic cells (NCCs), NK cells and CTL. NCCs are small and agranulocytes that spontaneously kill a variety of xenogeneic targets such as fish protozoan parasites. These cells were described in channel catfish (*Ictalurus punctatus*) (Graves *et al*., 1984; Evans and Jaso-Friedmann, 1992), rainbow trout (*Oncorhynchus mykiss*) (Greenlee *et al*., 1991), zebra fish (*Danio rerio*) (Jaso-Friedmann *et al*., 2002) and common carp (*Cyprinus carpio*) (Sakata *et al*., 2005). Catfish NCCs are defined by their reactivity with mAb 5C.6, which binds to the NCC receptor protein 1 (NCCRP-1) and functions as a pattern recognition receptor (Jaso-Friedmann *et al*., 1997; Evans *et al*., 1998; 1988). More recently, Kallio *et al*. (2011) have provided strong evidence that mammalian NCCRP-1 is a member of the lectin - type subfamily of F-box proteins.

In a previous study, we revealed a down-regulation of cytotoxic components (granzyme, NK lysin, FasL, IFNγ, Tbet, IL12b and CD4 mRNA) following chronic stress, in which NCCRP-1 mRNA, the marker of so-called NCC cells, increased fivefold. On the other hand, we observed CDγδ mRNA dominance in carp peripheral blood leukocytes and the levels of CD4 and CD8 mRNA were less than the total of CD3ξ mRNA. Furthermore,

the mRNA levels of CD56 and the receptors on NK cells, NILT1 (activator) and NILT2 (inhibitor) showed no change. Hence, we assumed that it is possible that there was no presence of NCC cells in carp leukocytes and that NCCRP-1 is probably used as ubiquitin, as revealed by Kallio *et al*. (2011). In cytotoxic cells in carp, probably additional cytotoxic cells exist as MAIT and γδT cells, as shown in mRNA levels (Shimon-Hophy and Avtalion, 2018; 2017). In order to establish our assumption, we used antibodies for: A. MR1, a marker to MAIT cells; b. CD3ε, a marker of T cells; c. TCRγδ, a marker of γδ T cells and d, NCCRP-1, a marker of so-called NCCs. Because carp has no specific antibodies to those markers, we used for the above markers antibodies with multi-species reactivity. The ability of these antibodies to bind to receptors in different animal species is probably due to the existence of a conserved core identified by these antibodies. Therefore, they may also react with corresponding carp cell receptors and indeed, these antibodies have been bound to corresponding carp receptors in this study. Even though this binding might not be highly efficient, but it still allows a picture of the carp leukocyte profile.

Materials and Methods

Animals

Common carp (150±30 g were obtained from a local fish farm (Maagan Michael, Israel). The fish were acclimatized to laboratory conditions at least one month before experiments. Fish were maintained in containers (105×105×80 cm) with air bobbling and recirculating freshwater at 24±2°C, in a 12-h light/12-h dark cycle and fed a commercial diet once a day. Two weeks before the experiment, the fish were kept in net cages (75×28×48 cm), 1 fish per cage. The cages were maintained in water tank (350×300×100 cm), equipped with a biological filter and continuous flow of water and air.

Cell Separation

About 1 ml. of blood was removed from the caudal vein of 6 fish by a heparinized syringe and diluted in 9 ml Dulbecco's Modified Eagle Medium (DMEM) solution (Biological Industries, Israel). After two weeks, the fish were treated for acute stress by exposure to air for 10 min and then immersion in water for 30 min. After three cycles of exposure/immersion, the fish were left for 24 h in water and then1ml blood was removed from the caudal vein of each fish by a heparinized syringe and diluted in 9 ml DMEM solution (Biological Industries, Israel). For chronic stress, the regime of exposure to air/immersion in water was done during three weeks, as detailed before (Shimon-Hophy and Avtalion, 2017). Leukocytes were separated on Ficoll-Paque™ plus (GE Healthcare). After three washes, a cell sample of each fish leukocytes was transferred to cover slips by cyto-centrifugation (Elliot-Shandon, Recyclab).

Cell Staining

Cover slips were added to 6 well plates. The cells were fixed by 4% Para formaldehyde (Sigma-Aldrich) for 30 min, washed 3 times with Phosphate Buffer Saline (PBS) pH 7.4 and then blocked by PBS containing 1% Bovine Serum Albumin (BSA) (Sigma-Aldrich) and 0.4% Triton X-100 (Bio-Lab, Jerusalem, Israel) for 45 min. The cells were washed 3 times with PBS and incubated with primary antibody (1:100): γ/δT cells - mouse monoclonal TCR gamma/delta (clone TCR1), which also reacts with chicken and reptiles (Novus Biologicals, Centennial CO USA); MAIT cells - mouse monoclonal anti MR1(clone 26.5),which also reacts with human, bovine and rats (EMD Millipore, Temecula, Ca, USA);T cells (CD3ε) – monoclonal rat anti-human CD3 Alexa Fluor 647 (clone CD3-12), which reacts with multi-species (Bio-Rad); and so-called NCC – monoclonal mouse anti-NCCRP-1 clone 5C.6 (Novus Biologicals, Centennial CO. USA), for overnight at 4°C. Cells were washed 3 times with PBS and then with PBS containing 0.2% Triton X-100 (PBST) followed by incubation with secondary antibody (1:200), chicken anti-mouse CF™ 568 (Sigma-Aldrich) for TCRg/d, NCCRP-1 and MR1 antibodies (Sigma-Aldrich), for 30 min at room temperature. Cells were washed 3 times with PBS and then 15 min with PBST and incubated with 4', 6-diamidino-2-phenylindole dihydrochloride (DAPI) (Invitrogen) according to manufacturer guide for 5 min and washed 3 times with PBS. Cover slips were transferred to slides and mounted with Fluoro-gel with DABCO™ (EMS, Hatfield, PA, USA).

Double staining: Cells were incubated overnight at 4°C with the antibody mouse anti-NCCRP-1 (clone 5C.6), washed 3 times with PBS and then 15 min with PBST, incubated with goat anti-mouse CF488 (Sigma-Aldrich)(1:300) and some samples with chicken anti-mouse CF568 (Sigma-Aldrich) (1:200) for 30 min at room temperature, washed 3 times with PBS and then 15 min with PBST and incubated with second primary antibody (2:100): Monoclonal rat anti-human CD3 Alexa Fluor 647 (clone CD3-12) (Bio-Rad), PE anti-human/mouse/rat MR1 Antibody (clone 26.5) (Biolegend, San Diego, Ca, USA) and FITC anti-mouse TCR gamma/delta (TCR1) (in a samples incubated with CF568 secondary antibody), for 2 h. Then, they were washed 3 times with PBS followed by PBST for 15 min and incubated for 5 min with DAPI at room

temperature. Cover slips were washed 3 times, transferred to slides and mounted with Fluoro-gel with DABCO™ (EMS, Hatfield, PA, USA). All the treatments after incubation with fluorescent antibodies were done in the dark.

Microscopy

Microscope used was an inverted Leica DMi8 scanning confocal microscope, driven by LASX software (Leica Microsystems, Mannheim, Germany). Excitation of DAPI was by a 405 nm laser (with PMT detector set on 414-464 nm). Other fluorophores were excited with a tunable white light laser set at 50% power, at the appropriate peak excitation wavelengths. Specifically: 492 nm (with HyD detector set at 502-582 nm, with time gating at 0.3-10 ns), 556 nm (with HyD detector set at 576-656 nm, with time gating at 0.1-10 ns) and 647 nm (with HyD detector set at 660-740 nm, with time gating at 0.1-10 ns). Objective used was an HC PL APO CS2 63x/1.40 OIL lens and scanning was set on a 4x line average.

Results

γδ T cells (TCR-gamma/delta) were present in 20-30% of leukocytes and T cell (CD3ε) levels were less than those of γδ T cells. MAIT (MR1) cells were about half of T cells. About a third of the leukocytes consisted of NCCRP-1. Cell count in the field of view of the microscope showed no significant difference by T-test on the number of cells during stress treatments (Table 1).

There was a change in cell surface area (up to 3-fold) during stress treatments. Some cell types had a prominent cytoplasm and more receptors in acute stress (Fig. 1B) as opposed to few receptors in the cytoplasm of leukocytes in chronic stress after the third week (Fig. 1C).

NCCRP-1 appeared in different types of cells and more than in one type. It was shown in CD3 cells as well as in MAIT (MR1) and γδ T cells (Fig. 2). Moreover, NCCRP-1 was present even in thrombocytes (Fig. 2).

Discussion

The use of antibodies with multi species reactivity confirms our assumption that there is a conserved core in the studied receptors and this core is also conserved in the common carp receptors. For that reason, these antibodies were bound to the corresponding receptors in the carp leukocytes (Table 1, Fig. 1 and 2). Cell count in the field of view of the microscope showed no significant difference by T-test on the number of cells during stress treatments (Table 1). Despite the inaccuracy in fluorescent cell counts in the field of view of the microscope sections, the results showed that γδ T cells (TCR-gamma/delta) were present in 20-30% of leukocytes and T cell (CD3ε) levels were less than those of γδ T cells (Table 1). This antibody binding to CD3ε and TCRγδ corroborated with our study on leukocyte mRNA levels (Shimon-Hophy and Avtalion, 2018). The ratio between CD3ε to TCRγδ in Table 1 (2:3) was relatively similar to the ratio between CD3ξ and CD3γδ mRNA (Shimon-Hophy and Avtalion, 2018). In this study, we showed also that CD4 and CD8 mRNA levels only accounted for a quarter of CD3γδ. Therefore, we assumed that there was an additional type of T cells (Shimon-Hophy and Avtalion, 2018). MR1 levels in Table 1 confirm this assumption: MAIT cells are prominent in T cells (about half). The microscopic picture (Fig. 1) ascertained the PCR picture (Shimon-Hophy and Avtalion, 2018), showing that γδ T cells are dominant in the carp lymphocyte population. Such dominance was also found in zebra fish (*Danio rerio*) (Hayday, 2000; Wan *et al*., 2017).

Microscopic viewing of cells revealed an increase of up to3-fold in cell surface area and receptors amount in some cell types following stress treatments (Fig. 1). In acute stress (Fig. 1 B), the cell surface area and the number of receptors were very high, whereas after 3 weeks of chronic stress, the cell surface area and the number of receptors were lower. This phenomenon supports our hypothesis that chronic stress might not change the overall number of cells but rather it change their activity, like in the case of down-regulation of IFNγ, IL12b, Tbet, IL8, granzyme, NK lysin, FasL mRNA production in cytotoxic cells and probably also the proliferation of Th1 and B cells (Shimon-Hophy and Avtalion, 2018; 2017). CD3ε in Figure 1C shows that some T cells appeared to be active, because their cell surface area increased 3-fold and the receptor number were high, whereas others had a small surface area with fewer receptors (Fig. 1C). This picture corroborates our study, showing that chronic stress down-regulates inflammatory, regulatory and cytotoxic cells. However, towards the third week of chronic stress, the regulatory and inflammatory cells recovered and went back to normal, whereas the cytotoxic cells were left down-regulated (Shimon-Hophy and Avtalion, 2018; 2017).

The picture in Figure 2 confirms our assumption that in the common carp leukocytes, the presence of unique cells like NCCs might not be shown and the NCCRP-1 should be considered as ubiquitin, as proved by Kalio (Kallio *et al*., 2011). The NCCRP-1 function as an ubiquitin explained the anomaly of the increase in its mRNA levels (fivefold) during chronic stress while many cytotoxic cytokines were down-regulated; a known phenomenon that increases vulnerability and mortality of fish (Shimon-Hophy and Avtalion, 2018).

It was interesting to find the presence of NCCRP-1 in thrombocytes too (Fig. 2). Since thrombocytes in teleost have a cell nucleus, they probably have a function in the catabolism of proteins; however, this is a matter for further study. Our findings contradict previous studies (Mali *et al*., 2017; Zheng *et al*., 2018) that claim that unique cells like NCC cells exist in teleost and are supported by the presence of NCCRP-1 in gilthead sea bream (*Sparus aurata*) lymphocytes, monocyte/macrophages and acidophilic granulocytes (Cuesta *et al*., 2005) and tilapia (*Oreochromis niloticus*) lymphocytes (Ishimoto *et al*., 2004). These findings confirm the presence of ubiquitin function in different cytotoxic cells. We assume that these studies (Mali *et al*., 2017; Zheng *et al*., 2018) investigating the so-called NCC cells, were mainly performed on γδ T cells because of their amount in lymphocytes and their nonspecific cytotoxic activity.

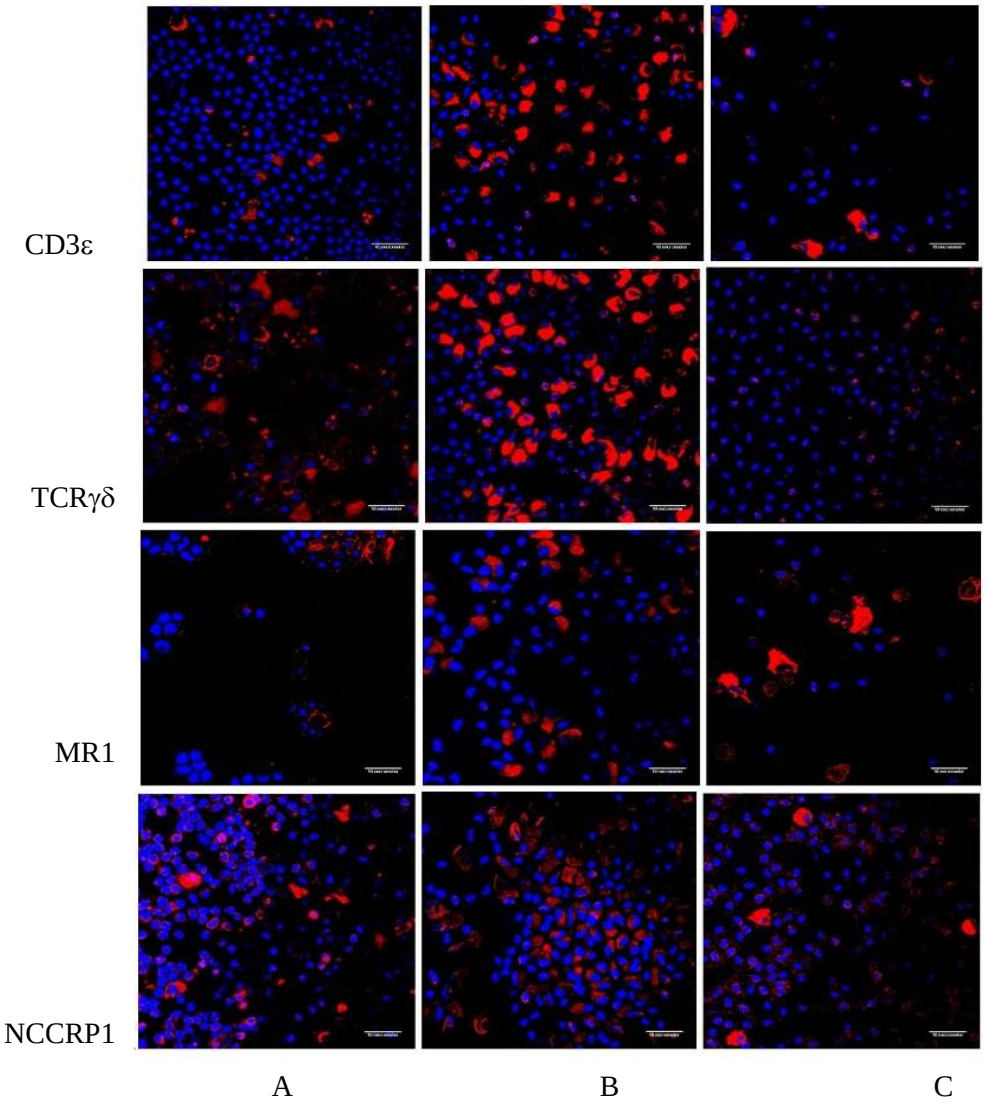

Fig. 1: Cell marker distribution in common carp peripheral blood leukocytes following stress treatments. 6 fish treated in acute (1 regime) and chronic (3 regimes/week) stress during 3 weeks while in each regime the fish were exposed to the air 3 times for 10 min. Leukocytes isolated by Ficole gradient from each fish. Slides were analyzed by an inverted Leica DMi8 scanning confocal microscope, driven by LASX software. Scale bar – 10 µm; magnification – X63 with oil. A – control, B – acute stress (1 regime of air exposure), C – chronic stress following 3 weeks (10 regimes of air exposure). Cells bounded to the antibodies: CD3ε (T cells) - Monoclonal rat anti human CD3 Alexa Fluor 647 (clone CD3-12) (red); TCRγδ (γδ T cells) - mouse monoclonal TCR gamma/delta (clone TCR1), MR1 (MAIT cells) - mouse monoclonal anti MR1 (clone 26.5), NCCRP1 - monoclonal mouse anti NCCRP1 (clone 5C.6). MR1, TCRγδ and NCCRP1 were bounded with a second antibody, chicken anti mouse CF^{TM}568 (red). Nucleus of all cells was stained by DAPI (blue).

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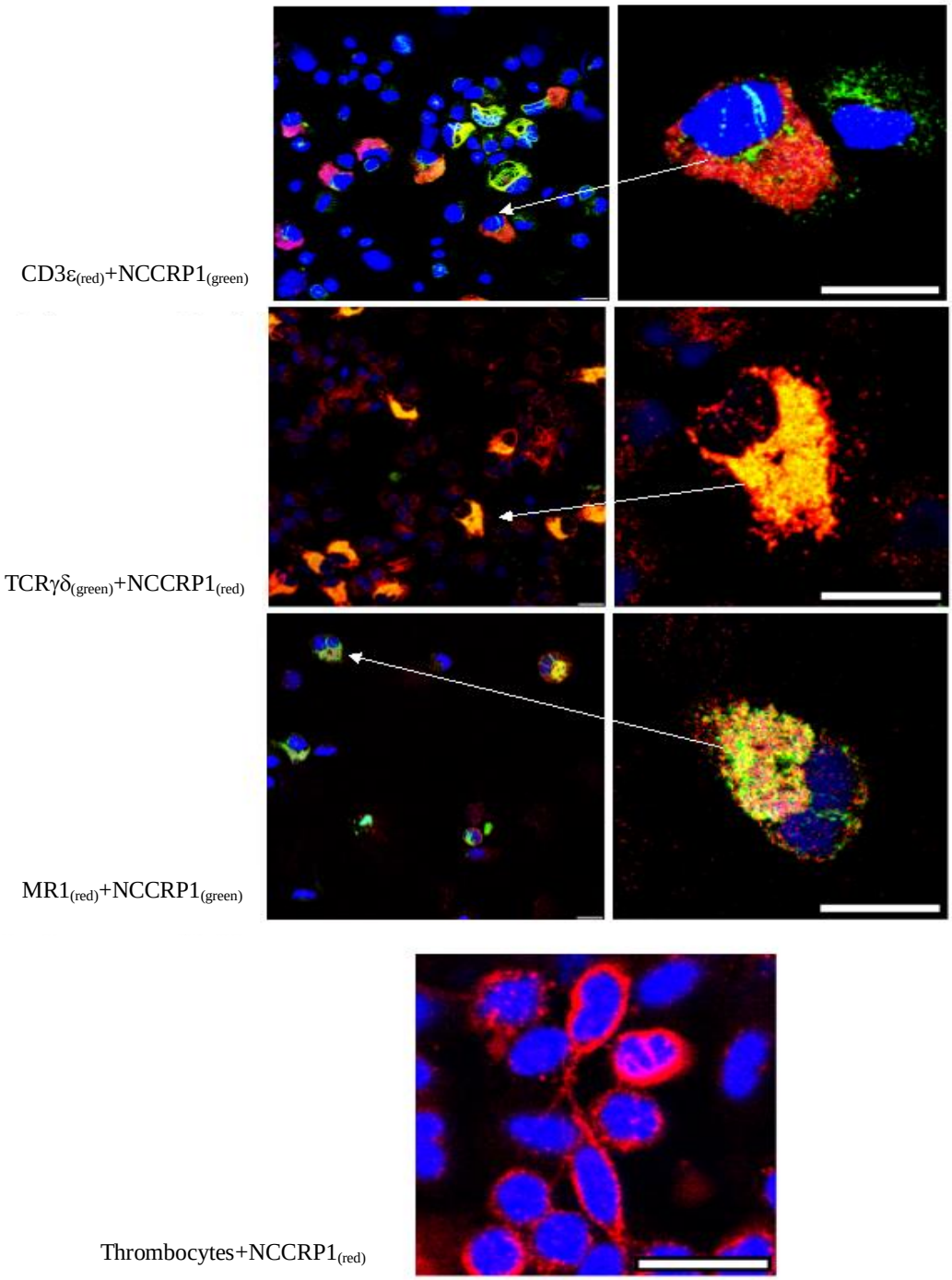

Fig. 2: Presence of NCCRP1 in T (CD3ε), MAIT (MR1), γδ T (TCRγδ) cells and thrombocytes in common carp peripheral blood leukocytes. Leukocytes isolated by Ficole gradient from 6 fish that were treated during one week by 4 air exposure regimes in which the fish were exposed 3 times for 10 min to the air. The pictures represent the findings from six fish. Scale bar = 10 μm. Slides were analyzed by an inverted Leica DMi8 scanning confocal microscope, driven by LASX software; magnification X63 with oil. Blue- cell nucleus (DAPI); Chromo fore fluorescent antibodies for: CD3ε+NCCRP1 – CD3ε, stained by Alexa Fluor 647 (red) and NCCRP1 stained by goat anti mouse CF488 (green); TCRγδ+NCCRP1- TCRγδ stained by FITC anti-mouse TCR gamma/delta (green) and NCCRP1 by chicken anti-mouse CF568 (red); MR1+NCCRP1 – MR1 stained by PE anti-human/mouse/rat MR1 (red) and NCCRP1 stained by goat anti-mouse CF488 (green); thrombocytes stained by chicken anti-mouse CF568 (red)

Table 1: Percentage of fluorescent cell markers in peripheral blood leukocytes of common carp following stress treatments

Cell marker	Control	Acute stress	CSW1	CSW2	CSW3
CD3ε	13±9	18±6	14±8	16±8	19±5
MR1	10±9	13±5	6±4	2±1	7±5
TCRγδ	22±7	22±12	19±9	28±12	26±8
NCCRP1	30±6	20±14	33±17	28±6	18±1

Results are a mean ± SEM of fluorescent common carp peripheral blood leukocytes that were observed in the microscope field of view by confocal microscope, $p > 0.05$ (T test). 6 fish were treated in acute (1 regime) and chronic (3 regimes/weeks) stress while in each regime the fish was exposed to air 3 times for 10 min at 30-min. intervals between exposures. CSW1 – chronic stress during 9 days (4 regimes of air exposure), CSW2 – chronic stress during 15 days (7 regimes of air exposure); CSW3 – chronic stress during 22 days (10 regimes of air exposure).

Conclusion

Based on the above findings, it can be concluded that:

a. NCCRP-1 exists in T, MAIT and γδ T cells and not in only one type of cells. Therefore, the presence of NCC cells should be considered
b. About 20-30% of carp peripheral blood leukocytes are γδ T cells and about half of T cells are MAIT cells.

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Author's Contribution

Mazal Shimon-Hophy: Planned and made all experiments, summarized the results and wrote the manuscript.

Ramy R. Avtalion: The idea of learning and comparing the effects of acute and chronic stresses in carp (*Cyprinus carpio*), an animal model currently used in our laboratory.

Avi Jacob: Assisted in analyzing microscopic images.

Ethics

This article is original and contains unpublished material. The corresponding author confirms that all of the other authors have read and approved the manuscript and no commercial or financial conflict of interests.

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