Archives of Immunology and Allergy

ISSN: 2639-1848

Volume 1, Issue 2, 2018, PP: 54-66

Chronic Stress Down Regulate IFNγ in Cytotoxic Cells of the Common Carp (*Cyprinus Carpio*)

Mazal Shimon-Hophy, Ramy R. Avtalion

Laboratory of Comparative Immunology and Genetics, The Mina & Everard Goodman Faculty of Life Sciences, Bar-Ilan University, Ramat-Gan, Israel.

hophymazal@gmail.com

***Corresponding Author:** Mazal Shimon-Hophy, Laboratory of Comparative Immunology and Genetics, The Mina & Everard Goodman Faculty of Life Sciences, Bar-Ilan University, Ramat-Gan, Israel.*

Abstract

The involvement of cytotoxicity in the negligible levels of IFNγ during chronic stress in common carp (Cyprinus carpio) was studied by measuring leukocyte levels using flow cytometry and immune components mRNA involved in cytotoxicity using real-time qPCR. Acute and chronic oxidative stresses were generated by different regimes of carp exposure to environmental air. Results revealed that CD4 and CD8a cell levels were both about 7% of the peripheral blood leukocytes, while the ratio between CD4 and CD3γ/δ mRNA was 1:4 and the ratio between CD3ξ and CD3γ/δ mRNA was 4:6.

Under conditions of acute stress, cytotoxicity did not take a part whereas, under conditions of chronic stress, the CD3γ/δ mRNA increased only in the first week (171%), while the mediator of proteolysis, NCCRP1 mRNA increased to peaks of 533%, and remained high during 22 days of chronic stress. The regulators of Th1 cells (CD4, CD3ξ, and Tbet mRNA) and the apoptotic cytokines (FasL, granzyme and NK- lysin mRNA) decreased continuously (up to 14%, 41%, 29%, 23%, 29%, and 26% respectively), during 22 days of chronic stress.

In conclusion, it is suggested that most of the lymphocytes in carp belong to the nonspecific immunity, while the γ/δ T cells are dominant. In acute stress, cytotoxicity probably does not participate in the 'fight or flight' process, while in the case of chronic stress; there is a deterioration of Th1 cells, suppression of the apoptotic processes as well in the total suppression of INFγ production.

Keywords: *cytotoxicity, gamma/delta T cell, acute/chronic stress, Th1 cell, NCCRP1, CD3zeta*

Introduction

Aquaculture conditions are often exposed to various stressors that increase fish sensitivity and expose them to various pathogens, resulting in high mortality that influences the feasibility of the aquaculture industry [1,2]. Stress influences humoral and cellular immune systems that are regulated by the nervous and endocrine systems via cytokines, hormones, neurotransmitters, and receptors, which are in constant communication to maintain homeostasis and orchestrate coordinated responses to imbalances and pathologies [3, 4].

In a previous study, we showed that in acute stress, the levels of IL1β, IL6, IL10, TNFa, and TGFβ mRNA increased and coped with the 'fight or flight' response in mammals [5]. However, in chronic stress, we revealed a sharp decrease in the levels of B lymphocytes, macrophages, as well as in the immune components IFNγ, IL12, IL8, and CD4. However, components of inflammatory, regulatory, and innate systems (complement C and IgM) did not change or temporarily changed and returned to homeostasis. We concluded that the null levels of IFNγ throughout chronic stress could not only be attributed to Th1-cell deterioration. Consequently, it was presumed that cytotoxic activities might be involved, although CD8a mRNA levels did not change significantly following chronic stress [5].

In mammals, cytotoxicity is mainly due to cytotoxic T lymphocytes (CTLs) and NK cells. These cells lyse

target cells by two mechanisms, namely, granule exocytosis and Fas ligand/Fas receptor (FasL/FasR) interaction, both of which require membrane contact with the target cells. Exocytosed granules contain pore-forming substances and other mediators. Pore formation in the outer membrane of target cells is induced by perforin, granulysin, and NK-lysin, allowing the entry of granzymes into the cytoplasm, and ultimately resulting in apoptotic cell death [6,7].

It has been reported that cytotoxicity in fish, as in mammals, can be specific and nonspecific. This has been reported in several teleost species, especially in cell cultures or cell lines of ginbuna crucian carp (*Carassiusc carassius*), channel catfish (*Ictalurus punctatus*), and rainbow trout (*Oncorhynchus mykiss*) [8,9]. These findings suggest the presence of three types of cells involved in nonspecific cytotoxicity in fish. The first is the nonspecific cytotoxic cell (NCC), as seen in the channel catfish. NCCs express a novel type of membrane protein, termed NCC receptor protein 1(NCCRP-1) that spontaneously kills a variety of xenogeneic targets [10,11]. NCC cells have been suggested to be the evolutionary precursor of mammalian NK cells [11]. The second, NK-like cell lines [12], not only kill the stimulating allogeneic cells, but also the unrelated allogeneic targets without prior sensitization, and express a putative Fc R, but not Ig or TCR. A novel immunoglobulin-like transcript (NILT) in teleost fish encodes polymorphic receptors with the cytoplasmic ITAM or ITIM and a new structural Ig domain similar to the natural cytotoxicity receptor NKp44 [13], known as a marker for activated human NK cells [14].These cells were also capable of killing virus-infected catfish cells [15,16]. The third type is the neutrophil. Carp and ginbuna neutrophils exhibited cytotoxic activity against multiple human tumor cells [17,18].

In contrast to the above, CTLs have a specific cytotoxicity. They recognize a specific antigen presented by class I MHC that triggers CTL immune response. They kill their cellular targets via two mechanisms, FasL/FasR and secretory granules consisting of perforin, granzymes, and NK - lysin. Each requires direct contact between the effector and target cells [19]. Both of these pathways induce apoptosis. A positive correlation exists between expressions of perforin/granzyme and activated mammalian CTLs [19,20,21]. Unlike mammalian NK cells and T cells, fish cytotoxic cells do not express membrane FasL unless soluble cytosolic FasL forms as have been detected thus far in tilapia, channel catfish (*Ictalurus punctatus*) [22,23], and gilthead seabream (*Sparus aurata*) [24]. It was stated that FasL secretion by activated NCCs can function in the presence of FasR positive target cells [25]. The present study strongly suggests that pathways of killing operative in fish are similar to those operative in mammals.

In the present work, we studied the influence of acute and chronic stress on cells and components of the cytotoxic response in common carp peripheral blood leukocytes in order to explain the null levels of IFNγ observed during chronic stress [5]. Although the tools are poor because of the absence of specific antibodies to many essential fish components, we could evaluate: (1). The cytotoxic cell marker levels of CD4+ [26], CD8+ [27], CD3γ/δ [28], CD3ξ [29], NK [30], macrophages (CD209, CD14) [31,32, 33], and NCC (NCCRP1) [11]; (2). The components regulating cytotoxicity by apoptosis as FasL and its receptor CD95, and the causing of cell lysis, NK-lysin and granzyme [19,20,21]; (3). The regulators of NK cell activity, NILT1 (activating receptor) and NILT2 (inhibitor receptor) [13]; and (4). The regulators of Th1 cell proliferation and maturation, STAT4, Tbet, and CXCR3 [26, 34] by purifying mRNA from stressed carp leukocytes, measuring immune components levels by PCR amplification and evaluating cell levels by fluorescence-activated cell sorting (FACS).

Materials and Methods

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

Stress Treatment

We used a group of 8 fish to follow changes in their individual peripheral blood immune components throughout the stress treatments. Therefore, control samples of 1 mL were taken with heparinized syringe from the caudal vein of each fish before stress treatments. Two weeks later, the fish were exposed for 10 min. to the air and then immersed for 30 min. in water. After three cycles of exposure/immersion; the fish were left for 24 h in the water [35, 36] and then 1 mL of blood was taken from the caudal vein of each fish with heparinized syringe. Two weeks later, the fish were treated for chronic stress during 3 weeks. The fish were similarly treated as in the acute stress group, but the exposures to the air and immersions, were repeated as above, three times a week for three weeks. Twenty four hours following the last air exposure, performed at the 9th, 16th and 23th days of chronic stress treatments, bloods have been taken from the caudal vein of 8 fish, as detailed above. Leukocytes from each blood sample were then separated on a Ficoll gradient.

Gene Expression Quantification

Total RNA was extracted from each blood sample with Hybrid-R™ Blood RNA kit according to the manufacturer's instructions (Geneall Biotechnology, Seoul, Korea). RNA quantification was carried out using a NanoDrop ND2000c spectrophotometer (Thermo Scientific). Total RNA quality was monitored by running samples on a 1.3% agarose gel. Adequate samples were used for complementary DNA (cDNA) synthesis which was carried out with the FastQuant RT Kit (with gDNase) (Tiangen, Beijing, China) and also served as a negative control for quantitative PCR (qPCR). Part of the cDNA was used for a standard curve in each qPCR experiment and the rest of the material was diluted to 100 ng µL−1. qPCR amplification was carried out in 20 µL reaction volume containing 5 µL of diluted cDNA (500 ng) used as a template for qPCR cytokine quantification, 10 µL iTaq™ Universal Syber Green Supermix (Bio Rad Laboratories, Hercules, Ca, USA) and 5 µL primer, resulting in a final concentration of 0.1 µM. All immune component samples were run in triplicate while standards (standard curve of each cytokine and of the RNA negative control following gDNase) in duplicate in the CFX96 (Bio Rad Laboratories, Hercules, Ca, USA) following the manufacturer's conditions, as follows: Initial denaturation for 30sec, 95°C, followed by 40 cycles of 5 sec denaturation at 95°C and 20/30 sec for annealing/extension at 57°C to 63°C (Table 1). The melting curve in each experiment was used to examine qPCR and primer quality. Results of qPCR experiment were accepted if: (1) There was no contamination of dimmers or other material; (2) the efficiency of the qPCR reaction was 90 to 109%, (3) the R line of the reaction was 0.98 to 1.

PCR Qualification

Amplification was performed in a 20 µL of a reaction volume containing 10 µL GoTaq Green Master Mix (Promega, Madison, WI, USA), 5 µL primer (in a final concentration of 0.1 µM) and 5 µL diluted cDNA (1500 ng). Samples were run in the UNO II (Biometric) as follows: Initial denaturation for 5 min at 95°C, followed by 30 cycles of 30 sec denaturation in 95°C, 30 sec annealing at 60°C and 30 sec. extension at 72°C, ending with 72°C for 10 min. Samples were loaded on an 1.3% agarose gel and visualized by a Gel DocTM XR+ imaging system (Bio Rad, Laboratories, Hercules, Ca, USA).

Primer Design

Primers were designed by the NCBI tool and purchased from Integrated DNA Technologies, Leuven, Belgium (IDT). Each primer was analyzed by an IDT Oligo Analyzer. Running conditions of each primer were analyzed and only those which showed negligible dimmer, high PCR efficiency (90-109%) and R≥0.98. were used (Table 1).

Data Analysis

All experiments were analyzed by the CFX96 (Bio-Rad) software. Ratio production of immune components between stress conditions to control was expressed as fold changes. Cq was normalized to gene reference 40S rRNA and analyzed according to the Pfaffl and Livak method [37, 38] by correcting the efficiency of each primer at stress relative to control.

Statistical Analysis

The acute stress results were tested for significance by paired F and T tests and those of chronic stress were analyzed by a one way ANOVA followed by Bonferroni and Tamhane Post Hoc Tests.

Table 1. *Primers for immune components and their annealing/extension temperature in the PCR and RT- qPCR reaction.*

cytokine		AET*	Product length	Forward	Reverse
CD3 γ/δ	DQ340867.1	60	105	ACGGTTGACACAGGAAGCAT	ACATGTGTGGAGTCCAGATGA
CD3ξ	XM_019075258.1	61	72	ATGACAGCACTTACACGCCT	GGCCTGCCTTCGTCTCAATC
CD4	DQ400124.1	59	120	CCATCAGTTATGCATCTGTCAAG	CTGGAAAAGGGGAGAGTTCTATT
CD8a	EU251078.1	63	96	TGTCCCTCTTCAATGGGAACT	ATGTAATGACGCCGGGTTGT
CD95	KJ174688.1	60	87	AATCCACAAGCACAGGGACC	TCCATAGCCAGAACACCACC
CD56	XM_019067984.1	100	60	GATCACACTAACTTGCGAGGC	AGTCCATGAAGCCTGCTCG
CD209	XM_019091138.1	60	118	AAAGAGGTTGGTTTTTCGTGTCC	ATGTGTCTCTGCTTCTCTTCACTC
CXCR3	XM_019088255.1	61	80	TAGCTGGAGCGTTGTTCTCG	AGCCAGGTATCGGTCAAAGC
FasL	KJ174689.1	61	88	CAGATCCTGAGGTTACAGACTGAAG	CAACTTGTCTCTGTGGAGTGATG
granzyme	GU362096.1	61	98	TGCAGCCGCTATTACAACGAA	CCGGAATCTCCCCAACAGGT
NCCRP1	AB186387.1	60	81	GCTGATGGTAAAACGGTCATCA	CTTTTTCCAGGTGTGTGAGTAGCA
NILT1	AJ811994.1	61	81	TATCGCTGTTGTTGTTGCTCA	CCAGATTTATAGGGGCATCTGATGT
NILT2	AJ811995.1	62	84	TGGTGTGGAGTGAAGAGGAGTTTAT	GCTGAAATATGATGGTTCTGCCTGT
NK-lysin	KX034213.1	57	80	GGAAGTTAGTGAACCAGTACACAG	GTTAGCACAGATGGTTCTGGCA
STAT4	KJ782030.1	59	95	CTGGCAGTTCTCAAGTTTTGC	CATTATAAGAAGCGTGTTGACCAAG
Tbet	EU729739.1	59	71	TGATCATAACGAAACAGGGACGG	TGTGGGGTCCAGTGAGTCGAT
40S	AB012087	61	83	TGGCGGACATACAGAACGAGAG	TCCTTCAACAGCGAGAACCC

AET, annealing/extension temperature*

Fluorescence-activated cell sorting (FACS)

About 1 mL blood was removed from the caudal vein of each 4 fish by a heparinized syringe and diluted in 9 mL Dulbecco's Modified Eagle Medium (DMEM) solution (Biological Industries, Israel). Leukocytes were separated on FicollPaque™plus (GE Healthcare). After three washes, cells were used for Flow Cytometry (FACS).

Cells were divided to 5 tubes, one for control and 4 for each antibody. The cells were incubated in PBS solution containing, 0.1% sodium azide and 2% Bovine Serum Albumin (BSA) (Sigma) and 3 µl of one of the antibodies anti fish Alexa Fluor 405 - CD4 (Novus Biologicals, Littleton, CO, USA), or anti fish DyLight 550 - CD8 (Novus Biologicals, Littleton, CO, USA), or anti fish Alexa Fluor 647 - NCAM-1/CD56 (Novus Biologicals, Littleton, CO, USA), or anti fish FITC - CD14 (Bio-Rad) for 30 min at 4°C, were washed twice and kept in a PBS solution containing 0.1% sodium azide, 2% BSA and 0.6% paraformaldehyde, at 4°C. Cell analysis was performed on a flow cytometer, FACS ARIA III (BD Bioscience) equipped with 4 lasers of 488 nm, 633 nm, 405 nm and 561 nm. The fluorescence Emission of each laser was collected through 530±30 nm, 660±20 nm, 450±40 nm, 582±15 nm respectively bandpass filters. About 1000000 cells within the gated region were identified. Results were analyzed by the FlowJo software (FlowJo, LLC, Ashland, Or, USA.).

Results

FACS results could not show levels of Th (CD3ξ), γ/δ T (CD3γ/δ), and NCC fish cells due to the absence of specific carp antibodies for the time being. At the same time, we were able to quantify by FACS, NK (CD56), macrophages/monocytes (CD14), CD8a, and CD4 common carp peripheral blood leukocytes using specific antibodies. A million gated cells were surveyed in each sample by FACS ARIA III, thus revealing 0.02%, 0.09%, 0.18%, and 5.51% cells respectively (Fig. 1).

mRNA markers of Th (CD3ξ), γ/δ T (CD3 γ/δ), CD4, CD8a, NK (CD56), macrophages/dendritic (CD209) cells, and NCC (NCCRP1) cells involved in cytotoxicity were distributed in the peripheral blood leukocytes of the common carp as follows: 15%, 25%, 6%, 8%, 0, 0, and 47%, respectively (Fig. 2).

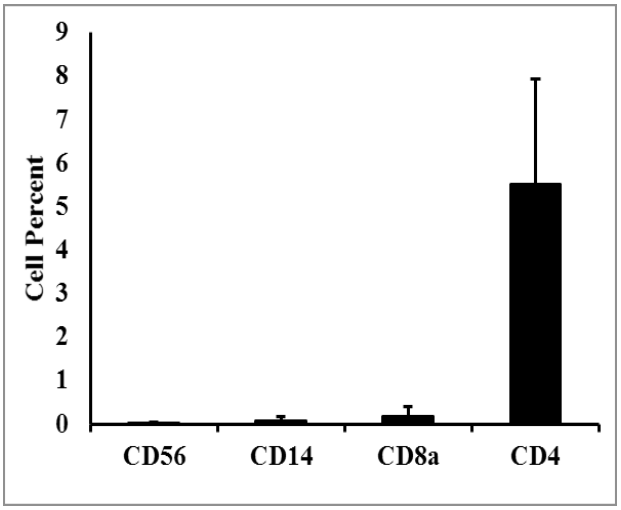

Fig 1. *Cell percent of common carp peripheral blood leukocytes labeled by Alexa Fluor 647 - anti fish NCAM-1/ CD56 antibody, FITC- anti fish CD14 antibody, DyLight 550- anti fish CD8a antibody, Alexa Fluor 405 - anti fish CD4 antibody. The results are mean of 4 fish ± SD. The leukocytes were bounded with anti-fish monoclonal antibodies and florescent cells percent was calculated from 1 million gated cells by FACS Aria III. FlowJo software used to analyze cell profile.*

Fig 2. *Cluster of differentiation mRNA levels in common carp leukocytes separated from peripheral blood of 8 fish by Ficole gradient. 1 - CD3ξ, 2 - CD3 γ/δ, 3 - CD4, 4 – CD8α, 5 - CD56, 6 – CD209, 7 – NCCRP1, 8 - reference gene 40S, M – cDNA marker of 100 bp size. Cluster of differentiations were produced from 2000 ng cDNA by PCR amplification and loaded on 1.3% agarose gel with TBE running solution*

During acute stress, by following the cell marker levels using qPCR, a significant decrease of about 50% in Th cells (CD3ξ mRNA) and an increase of up to 150% in NCC (NCCRP1 mRNA) cells were found (Table 2).

Table 2. *Cluster differentiation mRNA in peripheral blood leukocytes of common carp following acute stress.*

NCCRP1	CD8α	CD4	CD3 ξ	CD3 γ/δ	
1.00±0.45	1.00±0.44	1.00±0.41	1.00±0.23	1.00±0.13	ctrl
1.57±0.67*	1.16±0.56	0.92±0.37	0.52±0.06*	1.29±0.35	as

*, T test significance, p≤0.05. Each result represents a mean of 8 carp ± SEM. Acute stress was induced by a single regime of air exposure. Cell component cDNA levels were evaluated 24 h. after air exposure by qPCR amplification. Results were normalized to 40S rRNA and the ratio calculated by the ΔΔCq method. Ctrl - control; as - acute stress.

No change in the FasL mRNA and its receptor CD95 mRNA responsible for apoptosis could be seen (Table 3). NK - lysin and granzyme, responsible for the formation of apoptosis by creating pores in membranes and cell lysis, revealed that only NK-lysin mRNA decreased significantly up to 26% of control (Table 3). Regulators of NK cells NILT1, the activator receptor, and NILT2, the inhibitor receptor, showed that only NILT2 mRNA levels increased significantly up to 156% of the control level in acute stress (Table 3). The regulators of Th1 cell activity and proliferation, STAT4, Tbet, and the chemokine CXCR3, showed a significant decrease in the chemokine CXCR3 mRNA up to 83%, whereas the STAT4 mRNA increased significantly about 3 times that of the control. Tbet mRNA levels did not change (Table 3).

Table 3. *Cytotoxic cell components mRNA in peripheral blood leukocytes of common carp following acute stress.*

Tbet	STAT4	NK Lysin	NILT2	NILT1	granzyme	FasL	CXCR3	CD95	
1.00±0.41	1.00±0.63	1.00±0.61	1.00±0.31	1.00±0.81	1.00±0.39	1.00±0.17	1.00±0.38	1.00±0.28	ctrl
0.90±0.27	3.16±0.45*	0.26±0.07*	1.56±0.71*	1.77±0.57	0.45±0.11	1.00±0.19	0.83±0.21*	1.14±0.17	as

*, T test significance, p≤0.05. Each result represents a mean of 8 carp ± SEM. Acute stress was induced by a single regime of air exposure. Cell component cDNA levels were evaluated 24 h. after air exposure by qPCR amplification. Results were normalized to 40S rRNA and the ratio calculated by the ΔΔCq method. ctrl, control; as, acute stress.

During the third week of chronic stress, the cell markers of Th (CD3ξ) and CD4 cell mRNA decreased significantly and constantly to 41% and 14%, respectively (Fig. 3). CD3γ/δ mRNA (γ/δ T cells) increased significantly (up to 170%) only in the first week of chronic stress, and then returned to homeostasis. CD8α mRNA did not change significantly under chronic stress conditions for 22 days (Fig. 3).However, NCCRP1 mRNA (NCC cells) increased significantly up to 533% during the first week of chronic stress, before decreasing to 271% in the second week and 226% in the third week (Fig. 3).

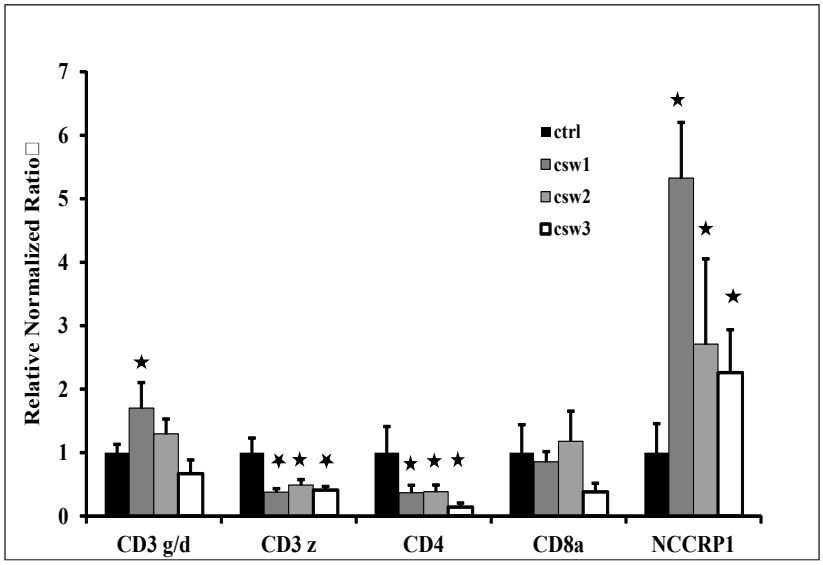

Fig 3. *The influence of chronic stress on cluster of differentiation mRNA levels in common carp leukocytes separated from peripheral blood of 8 fish by Ficole gradient*

* p≤0.05, results are mean ± SEM. Component levels were measured by qPCR after three air exposure regimes per week. Results were normalized to 40S rRNA and the component ratio to the control was calculated by the ΔΔCq method. ctrl, control fish; csw1, one week of chronic stress; csw2, two weeks of chronic stress; csw3, three weeks of chronic stress

CD95 and FasL mRNA, the receptor and its ligand, showed opposite trends. While CD95 mRNA increased significantly up to 338% of the control during the second week of chronic stress, before decreasing to 234% in the third week of chronic stress, the ligand FasL mRNA decreased significantly to 47% of the control level during the second week of chronic stress and continued to decrease to 23% during the third week (Fig. 4). The activator and inhibitor components of NK cells, NILT1 mRNA and NILT2 mRNA, did not show any significant change during 22 days of chronic stress. In addition, the chemokine CXCR3 mRNA, which attracts and promotes Th1 maturation, was not significantly influenced by chronic stress (Fig. 4). The cytokines involved in creating membrane pores and cell lysis, NK-lysin and granzyme, showed a significant decrease up to 30% and 26%, respectively, of the control level in the third week of chronic stress (Fig. 4). The transcription factor that participates in Th1 cell maturation and cytokine production, STAT4

mRNA, showed only a slight significant increase of up to 151% in the second week of chronic stress before returning to the control level. The transcription factor responsible for Th1 cell development and cytokine production, Tbet mRNA, decreased significantly up to 29% during the third week of chronic stress (Fig. 4).

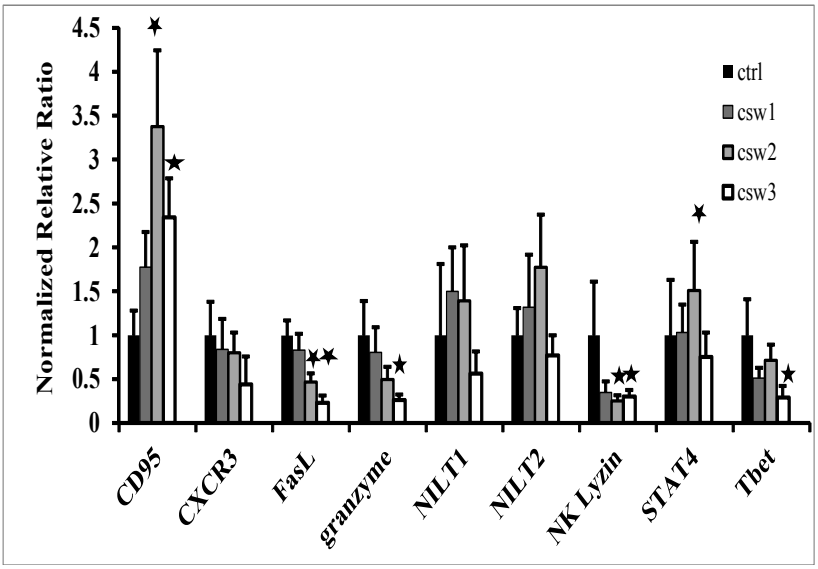

Fig 4. *Immune component mRNA levels during chronic stress. *, p<0.05 in one way ANOVA. Each result was a mean of 8 common carp peripheral blood ± SEM, Component levels were measured by qPCR after three air exposure regimes per week. Results were normalized to 40S rRNA and the component ratio to the control was calculated by the ΔΔCq method. ctrl, control fish; csw1, one week of chronic stress; csw2, two weeks of chronic stress; csw3, three weeks of chronic stress*.

Discussion

The leukocyte profile of carp peripheral blood (Figs. 1 and 2) showed clear differences from that of humans. In humans, lymphocytes CD4 and CD8 are mostly dominant, while the lymphocytes represent only about 30% of leukocytes [39, 40]. Consequently, the amount of CD4 and CD8 in humans represents about 20% of leukocytes. In carp, however, the amount of both cell types was less than 10% of peripheral blood leukocytes (Fig. 1) and the lymphocytes were about 70% of leukocytes [5]. Accordingly, most of the carp lymphocytes were nonspecific. This finding was strengthened by the ratio 1:4 between CD4 mRNA and CD3γ/δ mRNA (Fig. 2). The ratio between CD3ξ and CD3γ/δ mRNA was about 4:6 (Fig. 2) therefore, Th cells represented more than 10% of leukocytes and were probably nonspecific. These nonspecific Th cells are suggested to be the MAIT cells [41, 42] rather than NCC mainly because of the negligible levels of CD56 mRNA versus the high levels of CD3ξ mRNA (Fig. 1 and Fig. 2). We assume that the so-called NCC cells [11, 43] could be considered as γ/δ T [44,45,46] or MAIT cells [7,48,49] or both mainly because of the similarity in their activity rather than unique immune NCC cells only in fish. The protein NCCRP1, identifying the so-called NCC cells, seems to be a component present in those nonspecific cytotoxic cells. NCCRP1 has a function similar to that of the F-box proteins, which are known to bind protein substrates for ubiquitin - mediated proteolysis [50,51]. However, in order to establish this assumption, it is necessary to develop fish antibodies specific to the MAIT and γ/δ T cells and to NCCRP1.

The higher levels of CD3γ/δ mRNA versus the lower levels of CD4 and CD8a mRNA in the peripheral blood of the common carp (Fig. 2) were in line with the findings in zebrafish [46, 52] but slightly different from those in the sea bass, *Dicentrarchus labrax* [53]. The distribution of γ/δ T cells in carp as shown in Fig. 2, ruminants [54, 55, 56] and poultry [57] raises the question of whether this frequency of cells is a matter of "developed" or "undeveloped" immune system.

In acute stress, only CD3ξ mRNA, i.e., Th cells, decreased significantly up to 52% (Table 2). Therefore, it is possible that the nonspecific Th cells are responsible for that decrease. On the other hand, NCCRP1 mRNA

increased significantly (Table 2) and followed an elevation of proinflammatory and regulatory cytokines [5]. This elevation was in agreement with the findings of overcrowding stress in Solea senegalensis [58] and Sparus aurata [59]. The cytokines under acute stress, as summarized in Table 3, showed that there was no change in the apoptotic constituent levels of FasL mRNA and its receptor CD95 mRNA, and that there was probably no apoptotic activity. However, because there was no change in the levels of the transcription factor Tbet, while significant slight decrease in CXCR3 mRNA levels, was evident, it is possible that there was no change in Th1 activity. The receptors controlling NK cell activity showed that the level of the inhibitory NILT2 mRNA increased significantly to 156%; while the activator receptor NILT1 mRNA, stayed unchanged. This may indicate that the NK cells did not take part in acute stress. The mRNA levels of the cytotoxic components in the granules of cytotoxic cells, namely the pore forming, NK-lysin, decreased significantly to 26% of the control levels, while the component leading to apoptosis, the granzyme, decreased, but not significantly enough. It is possible that the NK lysin and granzyme decline was intended to block their involvement in the acute stress process. Although NK-lysin and granzyme work together in the elimination of cells, the different decline during acute stress may point on additional independent functions [60, 61]. The sharp elevation of the transcription factor STAT4 mRNA by 3 fold of the control levels (Fig. 4) might explain the increase in the levels of inflammatory and regulatory cytokines under acute stress [5, 62].

In the case of chronic stress, there was a significant decrease in the cells of the specific-immunity CD4 and Th cells (CD3ξ); their mRNA levels reached the lowest level after 22 days of stress, up to 14% and 41%, respectively (Fig. 3). These results were accompanied by a reduction of Tbet mRNA of up to 29% of control, while the CXCR3 mRNA level showed no significant changes (Fig. 4). These parallel findings confirm the damage of Th1 cells, as reported in previous studies [63,64,65,66]. CD3γ/δ mRNA, i.e., γ/δ T cells, increased significantly only in the first week of chronic stress up to 170% of control before returning to its initial level (Fig. 3). This raises the question of whether the increase in γ/δ T cells in the first week of chronic stress was associated with the suppression of cell activity of Th1 observed in this study (Figs. 3 and 4) and of B cells, plasma cells, and macrophages observed in previous experiments [5, 67, 68]. In parallel, NCCRP1 mRNA levels increased significantly fivefold of control in the first week, and slowly declined to two fold during the third week of chronic stress (Fig. 3). It is possible that γ/δT/ MAIT/ NK cells are responsible to the NCCRP1 mRNA levels, which is known to be involved in the breakdown of proteins [50, 51]. The possibility that this protein is involved in the suppression of the immune system under chronic stress is a matter of complementary study. The receptors controlling NK cell activity, NILT1 and NILT2 mRNA, revealed no change throughout chronic stress; moreover, we could not distinguish CD56 in either FACS or PCR in the control but in chronic stress during the second and third week, bands of CD56 were present (not yet published). If that phenomenon is involved in the suppression of specific immunity during chronic stress, it is a matter for further study. Consequently, we assume that Th1 and NK cells are likely to be inactive and, therefore, do not produce IFNγ. Moreover, the γ/δ T cells are also known to produce IFNγ [69, 70, 71]; however the results shown in Figure 3 indicate that the cells were not damaged, on the contrary, their amount increased. We concluded that the sharp drop in IFNγ levels during chronic stress [5] might be associated with the suppression of the production process in all cell types, even in the MAIT cells, which are known as producing IFNγ [72,73].

The apoptotic components FasL, NK-lysin, and granzyme mRNA were significantly depleted to the lowest levels on day 22 of chronic stress (Fig. 4).The decrease in FasL mRNA is in line with the findings of hypoxic stress in mice [74]. Moreover, the decrease in NK-lysin and granzyme followed NK cell cytotoxicity and perforin suppression under conditions of physical and psychological depression in humans [75]. This depletion, together with previous findings that revealed a decrease in IFNγ, macrophages, B cells and plasma cells [5], can explain the sensitivity to diseases under chronic stress. The elevation in STAT4 mRNA during the second week of chronic stress might explain the increase of regulatory and inflammatory cytokine levels [5] and probably not Th1 maturation and IFNγ production.

Conclusions

Based on the current results we can conclude that in common carp: (1). The peripheral blood lymphocytes profile revealed that the most part of these cells were nonspecific, i.e. CD4 and CD8 cells were only about 7% whereas the γ/δ T cells were dominant. (2). The main question of this study was what are the factors leading to the null levels of IFNγ during chronic stress as seen in our previous study [5]. The results of this study showed that the levels of the IFNγ producing cells namely γ/δ T, NK and MAIT cells which probably did not decreased. Therefore this may lead to the conclussion that the production process of IFNγ was suppressed. (3). In contrast to acute stress, the chronic stress influenced cytotoxicity expressed in deterioration of Th1 cells due to the decrease in CD4, CD3ξ and Tbet mRNA levels. (4). The apoptotic process throughout chronic stress was probably suppressed because of a sharp decrease in FasL, granzyme and NK lysin mRNA levels. (5). The sharp increase in NCCRP1 mRNA levels throughout chronic stress and of CDγ/δ mRNA in the first week of chronic stress question the probability of their involvement in the deterioration of Th1 cells as seen in the current study and of macrophages, B cells and plasma cells as seen in our previous study [5].

Acknowledgement

The authors would like to thank Yuri Kaminer from Marine Lab for his assistance in all statistic tests and Ahikam Gissis from Mishmar HaSharon Fishery for donating the carp.

References

[1] Franco R, Sanchez-Olea R, Reyes-Reyes EM, Panayiotidis MI. Environmental toxicity, oxidative stress and apoptosis: Menage a Trois. Mutat. Res., 2009; 674: 3-22. Available from: DOI: 10.1016/j.mrgentox.2008.11.012.

[2] Saeij JP, Verburg-van Kemenade LB, van Muiswinkel WB, Wiegertjes GF. Daily handling stress reduces resistance of carp to Trypano plasma borreli: in vitro modulatory effects of cortisol on leukocyte function and apoptosis. Dev. Comp. Immunol., 2003; 27: 233-245.Available from: DOI: 10.1016/S0145-305X(02)00093-9.

[3] Nardocci G, Navarro C, Cortes PP, Imarai M, Montoya M. et al. Neuroendocrine mechanisms for immune system regulation during stress in fish. Fish Shellfish Immunol.2014; 40: 531-538. Available from: DOI: 10.1016/j.fsi.2014.08.001.

[4] Tort L. Stress and immune modulation in fish. Dev Comp. Immunol., 2011; 35: 1366-1375. Available from: DOI: 10.1016/j.dci.2011.07.002.

[5] Shimon-Hophy M, Avtalion RR. Humoral and cellular effects of stress-An extensive model system. Am. J. Immunol 2017, 13 (2): 131-143. Available from: DOI: 10.3844/ajisp. 2017. 131.143.

[6] Smyth MJ, Kelly JM, Sutton VR, Davis JE, Browne KA, Sayers TJ, et al. Unlocking the secrets of cytotoxic granule proteins. J Leukoc.Biol. 2001; 70 (1):18-29.

[7] Trapani JA, Smyth MJ. Functional significance of the perforin/granzyme cell death pathway. Nat. Rev. Immunol 2002; 2:735.

[8] Nakanishi T, FischerU, Dijkstra JM, Hasegawa S, Somamoto T, Okamoto N,et al. Cytotoxic T cell function in fish. Dev. Comp. Immunol. 2002; 26: 131-139.

[9] Fischer U, Utke, K, Somamoto T, Kölner B, Ototake M, Nakanishi T.Cyto-toxic activities of fish leucocytes. Fish Shellfish Immunol. 2006; 20, 209–226.

[10] Evans DL, Jaso-Friedmann L, Smith Jr EE, St John A, Koren HS, Harris DT. Identification of a putative antigen receptor on fish nonspecific cytotoxic cells with monoclonal antibodies. J. Immunol. 1988; 141: 324-332.

[11] Evans DL, Jaso-Friedmann L. Nonspecific cytotoxic cells as effectors of immunity in fish. Annu.Rev. Fish Dis. 1992; 2: 109-121.

[12] Shen L, Stuge TB, Bengten E, Wilson M, Chinchar VG, Naftel JP, et al. Identification and characterization of clonal NK-like cells from channel catfish (*Ictalurus punctatus*). Dev. Comp. Immunol. 2004; 28: 139-152.

[13] Stet RJ, Hermsen T, Westphal AH, Jukes J, Engelsma M, LidyVerburg-vanKemenade BM, et al. Novel

immunoglobulin-like transcripts in teleost fish encode polymorphic receptors with cytoplasmic ITAM or ITIM and a newstructural Ig domain similar to the natural cytotoxicity receptor NKp44. Immunogenetics. 2005; 57(1-2): 77-89.

[14] Marras F, Bozzano F, De Maria A. Involvement of activating NK cell receptors and their modulation in pathogen immunity. J Biomed Biotechnol. 2011: 152430: 1-11.

[15] Yoshida SH, Stuge TB, Miller NW, Clem, LW. Phylogeny of lymphocyte heterogeneity: cytotoxic activity of channel catfish peripheral blood leukocytes directed against allogeneic targets. Dev. Comp. Immunol. 1995; 19: 71-77.

[16] Hogan RJ, Stuge TB, Clem LW, Miller NW, Chinchar VG. Anti-viral cyto-toxic cells in the channel catfish (*Ictalurus punctatus*). Dev. Comp. Immunol. 1996; 20: 115-127.

[17] Kurata O, Okamoto N, Ikeda Y. Neutrophilic granulocytes in carp *Cyprinuscarpio*, possess a spontaneous cytotoxic activity. Dev. Comp. Immunol. 1995; 19: 315-325.

[18] Kurata O, Hasegawa S, Okamoto N, Nakanishi T, Ikeda Y. Spontaneous cytotoxic activity of neutrophilic granulocytes in ginbuna crucian carp and channel catfish. Fish Pathol.1996; 31: 51-52.

[19] Chavez-Galan L, Arenas-Del Angel MC, Zenteno E, Chavez R, Lascurain R. Cell death mechanisms induced by cytotoxic lymphocytes. Cell Mol. Immunol, 2009; 6 (1): 15-25.

[20] Milstein O, Hagin D, Lask A, Reich-Zeliger S, Shezen E, Ophir E, et al. CTLs respond with activation and granule secretion when serving as targets for T-cell recognition. Blood. 2011; 117 (3):1042-1052. Available from: doi:10.1182/blood-2010-05-283770.

[21] Ordás MC, Cuesta A, Mercado L, Bols NC, Tafalla C. Viral hemorrhagic septi-caemia virus (VHSV) up-regulates the cytotoxic activity and the perforin/granzyme pathway in the rainbow trout RTS11 cell line. Fish Shellfish Immunol. 2011; 31(2): 252-259.

[22] Bishop GR, Taylor S, Jaso-Friedmann L, Evans DL. Mechanisms of nonspecific cytotoxic cell regulation of apoptosis: cytokine-like activity of Fas ligand. Fish Shellfish.Immunol. 2002; 13 (1): 47-67.

[23] Kaur H, Jaso-Friedmann L, Evans DL. Single base oligodeoxy guanosine upregulates Fas ligand release by nonspecific cytotoxic cells. Dev. Comp. Immunol. 2004; 28 (6): 571-579.

[24] Cuesta A, Esteban MA, Meseguer J. Identification of a FasL-like molecule in leucocytes of the teleost fish gilthead sea bream (*Sparus aurata L.*). Dev Comp. Immunol. 2003; 27(1): 21-27.

[25] Picchietti S, Guerra L, Bertoni F, Randelli E, Belardinelli MC, Buonocore F ,et al. Intestinal T cells of *Dicentrarchus labrax* (L.): gene expression and functional studies. Fish Shellfish Immunol. 2011; 30: 609-617.

[26] Zhu J, Paul WE. CD4 T cells: fates, functions, and faults. Blood. 2008; 112: 1557-1569; Available from: doi.org/10.1182/blood-2008-05-078154.

[27] Hoyer S, Prommersberger S, Pfeiffer IA, Schuler-Thurner B, Schuler G, Dörrie J, Schaft N. Concurrent interaction of DCs with CD4(+) and CD8(+) T cells improves secondary CTL expansion: It takes three to tango. Eur. J. Immunol. 2014; 44(12): 3543-59. Available from:doi:10.1002/eji.201444477.

[28] Vantourout P, Hayday A. Six-of-the-best: unique contributions of γδ T cells to immunology. Nat Rev. Immunol. 2013; 13(2): 88-100.Available from: doi: 10.1038/nri3384.

[29] Weissman AM, Hou D, Orloff DG, Modi WS, et al. Molecular cloning and chromosomal localization of the human T-cell receptor zeta chain: distinction from the molecular CD3 complex. Proceedings of the National Academy of Sciences of the. USA. 1988; 85: 9707-9713.

[30] Vivier E, Raulet DH, Moretta A, Caligiuri MA, Zitvogel L, Lanier LL, et al. Innate or adaptive immunity? The example of natural killer cells. Science. 2011; 331 (6013): 44- 49. Available from: doi:10.1126/science.1198687.

[31] McGreal E, Miller J, Gordon S. "Ligand recognition by antigen-presenting cell C-type lectin receptors". Curr.Opin.Immunol. 2005;

17 (1): 18-24. Available from: doi:10.1016/j.coi.2004.12.001.

[32] Viriyakosol S, Mathison JC, Tobias PS, Kirkland TN. Structure-Function Analysis of CD14 as a Soluble Receptor for Lipopolysaccharide. J.Biol. Chem. 1999; 275(5): 3144-3149.

[33] Kirkland, T.N., Viriyakosol, S., Structure-function analysis of soluble and membrane-bound CD14. Prog.Clin.Biol. Res. 1998; 397: 79-87.

[34] Qin S, Rottman JB, Myers P, Kassam N, Weinblatt M, Loetscher M, et al. The chemokine receptors CXCR3 and CCR5 mark subsets of T cells associated with certain inflammatory reactions. J. Clin. Invest. 1998; 101 (4): 746-754. Available from: doi:10.1172/JCI1422.

[35] Melamed O, Timan B, Avtalion RR., Noga EG. Design of a stress model in the hybrid bass (Morone Saxatilis X Morone Crysops). Isr. J. Aquacult., 1999; 51: 10-16.

[36] Dror M, Sinyakov MS, Okun E, Dym M, Sredni B, et al. Experimental handling stress as infection-facilitating factor for the goldfish ulcerative disease. Vet.Immunol.Immunopathol. 2006; 109: 279-287. Available from: DOI: 10.1016/j.vetimm.2005.08.022.

[37] Livak KJ, Schmittgen TD. Analyzing real time PCR data by the comparative CT method. Nat. Protocols, 2008; 3: 1101-1108.Available from: DOI: 10.1038/nprot.2008.73.

[38] Pfaffl MW. 2001. A new mathematical model for relative quantification in real-time RT–PCR. Nucleic Acids Res., 2001; 29: 2002-2007.Available from: DOI: 10.1093/nar/29.9.e45.

[39] Uppal SS, Verma S, Dhot PS. Normal Values of CD4 and CD8 Lymphocyte Subsets in Healthy Indian Adults and the Effects of Sex, Age. Ethnicity, and Smoking. Cytometry Part B (Clin. Cytometry). 2003; 52B:32-36.

[40] Jiang W, Kang L, Lu H-Z, Pan X, Lin Q, Pan Q, Xue Y, et al. Normal Values for CD4 and CD8 Lymphocyte Subsets in Healthy Chinese Adults from Shanghai. Clin.Diagn.Lab.Immunol. 2004; 11(4): 811–813. Available from: DOI: 10.1128/CDLI.11.4.811–813.2004.

[41] Napier RJ, Adams EJ, Gold MC, Lewinsohn DM. The Role of Mucosal Associated Invariant T Cells in Antimicrobial Immunity. Front.Immunol. 2015; 6: 344-353. Available from: doi:10.3389/fimmu.2015.00344.

[42] Gold MC, Lewinsohn DM. Mucosal associated invariant T cells and the immune response to infection.. Microbes infect. 2011; 13: (8–9): 742-748. Available from: doi:10.1016/j.micinf.2011.03.007.

[43] Evans DL, Leary JH 3rd, Jaso-Friedmann L. Nonspecific cytotoxic cells and innate immunity: regulation by programmed cell death. Dev Comp Immunol. 2001; 25(8-9): 791-805.

[44] Holtmeier W, Kabelitz D. Gamma delta T cells link innate and adaptive immune responses. Chem. Immunol.Allergy. 2005; 86: 151-83. Available from: doi:10.1159/000086659.

[45] Born WK, Reardon CL, O'Brien RL. The function of gamma delta T cells in innate immunity. Curr. Opin.Immunol. 2006; 18 (1): 31-38. Available from: doi:10.1016/j.coi.2005.11.007.

[46] Hayday AC. [gamma][delta] cells: a right time and a right place for a conserved third way of protection. Ann Rev Immunol. 2000; 18: 975-1026.

[47] Kurioka A, Walker LJ, Klenerman P, Willber CB. MAIT cells: new guardians of the liver. Clin.Trans. Immunol. 2016; 5: 1-11.

[48] Wong EB, Ndung'u T, Kasprowicz VO. The role of mucosal-associated invariant T cells in infectious diseases. Immunol. 2016; 150, 45-54.Available from: doi:10.1111/imm.12673.

[49] Hinks TSC. Mucosal-associated invariant T cells in autoimmunity, immune-mediated diseases and airways disease. Immunol. 2016; 148, 1–12. Available from: doi:10.1111/imm.12582.

[50] Kipreos ET, Pagano M.The F-box protein family. Genome Biol. 2000; 1(5): reviews 3002.1-3002.7.

[51] Kallio H, Tolvanen M, Ja ¨nis J, Pan P-w, Laurila E, et al. (2011) Characterization of Non-Specific Cytotoxic Cell Receptor Protein 1: A New Member of the Lectin-Type Subfamily of F-Box Proteins.

PLoS ONE. 2011; 6(11): e27152. doi:10.1371/journal.pone.0027152.

[52] Wan F, Hu C-B, Ma J-X, Gao K, Xiang L-X, Shao, J-Z. Characterization of γδ T Cells from Zebra fish provides insights into their important role in adaptive humoral immunity. Front. Immunol.2017; 7:675.Available from: doi: 10.3389/fimmu.2016.00675.

[53] Buonocore F, Castro R., Randelli E, Lefranc M-P, Six A, et al. Diversity, Molecular Characterization and Expression of T Cell Receptor c in a Teleost Fish, the Sea Bass (*Dicentrarchus labrax*, L). PLoS ONE. 2012; 7(10): e47957. Available from: doi:10.1371/journal.pone.0047957.

[54] Hein WR, Mackay CR. Prominence of gamma delta T cells in the ruminant immune system. Immunol.Today.1991; 12: 30-34.

[55] Mackay CR, Hein WR.A large proportion of bovine T cells express the gamma delta T cell receptor and show a distinct tissue distribution and surface phenotype. Int.Immunol. 1989; 1(5): 540-545.

[56] Sinkora M, Butler JE. The ontogeny of the porcine immune system. Dev.Comp.Immunol. 2009; 33: 273-283.

[57] Sowder JT, Chen CL, Ager LL, Chan MM, Cooper, MD. A large subpopulation of avian T cells expresses a homologue of the mammalian T gamma/delta receptor. J.Exp.Med., 1988; 167: 315-322.

[58] Tapia-Paniagua ST, Vidal S, Lobo C, Prieto-Álamo MJ, Jurado J, Cordero H, et al. The treatment with the probiotic Shewanella putrefaciens Pdp11 of specimens of *Solea senegalensis* exposed to high stocking densities to enhance their resistance to disease. Fish & Shellfish Immunol.2014; 41:209-21.

[59] Cordero H, Morcillo P, Cuesta A, Brinchmann MF, Esteban MA. Differential proteome profile of skin mucus of gilthead seabream (*Sparus aurata*) after probiotic intake and/or overcrowding stress. J.Proteom. 2016; 132: 41–50.

[60] Pereiro P, Romero A, Díaz-Rosales P, Estepa A, Figueras A, Novoa B. Nucleated Teleost Erythrocytes Play an Nk-Lysin- and Autophagy-Dependent Role in Antiviral Immunity. Front. Immunol.2017; 8:1458. Available from: doi: 10.3389/fimmu.2017.01458.

[61] Lieberman J. Granzyme A activates another way to die. Immunol Rev. 2011; 235(1): 93–104.

[62] Shen X-D, Ke B, Zhai Y, Gao F, Anselmo D, Lassman CR, et al. Stat4 and Stat6 signaling in hepatic ischemia/reperfusion injury in mice: HO-1 dependence of Stat4 disruption - mediated cytoprotection. Hepathology, 2003; 37: 37(2): 296- 303.

[63] Zhu J, Yamane H, Paul WE. Differentiation of effector CD4 T cell populations. Annu. Rev. Immunol., 2010; 28: 445-489.

[64] Abimannan T, Peroumal D, Parida JR, Barik PK, Padhan P, Devadas S. Oxidative stress modulates the cytokine response of differentiated Th17 and Th1 cells. Free RadicBiol Med. 2016; 99: 352-363. Available from: doi: 10.1016/j.freeradbiomed.2016.08.026.

[65] Szabo SJ, Sullivan BM, Peng SL, Glimcher LH.Molecular mechanisms regulating Th1 immune responses. Annu Rev Immunol, 2003; 21: 713- 758.

[66] Wang T, Holland JW, Martin SA, Secombes CJ. Sequence and expression analysis of two T helper master transcription factors, T-bet and GATA3, in rainbow trout *Oncorhynchus mykiss* and analysis of their expression during bacterial and parasitic infection. Fish.Shell.Immunol. 2010; 29(5): 705 – 715. Available from: DOI: 10.1016/j.fsi.2010.06.016.

[67] Ke Y, Pearce K, Lake JP, Ziegler HK, Kapp JA. Gamma delta T lymphocytes regulate the induction and maintenance of oral tolerance. J Immunol, 1997; 158: 3610-3618.

[68] Locke NR, Stankovic S, Funda DP, Harrison LC. TCR gamma delta intraepithelial lymphocytes are required for self-tolerance. J Immunol.2006; 176: 6553-6559.

[69] Skeen MJ, Ziegler HK. Activation of gamma delta T cells for production of IFN-gamma is mediated by bacteria via macrophage-derived cytokines IL-1 and IL-12. J Immunol.1995; 154(11):5832-41.

[70] Yin Z, Chen C, Szabo S J, Glimcher LH, Ray A, Craft J. T-Bet expression and failure of GATA-3 cross-regulation lead to default production of IFN-gamma by gamma delta T cells. J. Immunol. 2002; 168: 1566-1571.

[71] Yin Z, Zhang DH, Welte T, Bahtiyar G, Jung S, Liu L, et al. Dominance of IL-12 over IL-4 in gamma delta T cell differentiation leads to default production of IFN-gamma: failure to down-regulate IL-12 receptor beta 2-chain expression. J.Immunol. 2000; 164: 3056-3064.

[72] Sundström P, Ahlmanner F, Akéus P, Sundquist M, Alsén S, Yrlid U, et al. Human Mucosa-Associated Invariant T Cells Accumulate in Colon Adenocarcinomas but Produce Reduced Amounts of IFN-γ. J Immunol. 2015; 195(7): 3472-3481. Available from: doi: 10.4049/jimmunol.1500258.

[73] Shaler CR, Tun-Abraham ME, Skaro AI, Khazaie K, Corbett AJ, Mele T, et al. Mucosa-associated invariant T cells infiltrate hepatic metastases in patients with colorectal carcinoma but are rendered dysfunctional within and adjacent to tumor microenvironment. Cancer Immunol. Immunother.2017; 66:1563–1575.Available from: DOI 10.1007/s00262-017-2050-7.

[74] Liu Y, Pop R, Sadegh, C, Brugnara C, Haase VH, Socolovsky M. Suppression of Fas-FasL coexpression by erythropoietin mediates erythroblast expansion during the erythropoietic stress response in vivo. Blood. 2006; 108:123-133. Available from: doi.org/10.1182/blood-2005-11-4458.

[75] Duggal NA, Upton J, Phillips AC, Hampson P, Lord JM. NK cell immunesenescence is increased by psychological but not physical stress in older adults associated with raised cortisol and reduced perforin expression. Age. 2015; 37(1): 11–15. Available from: doi: 10.1007/s11357-015-9748-2.

Citation: **Mazal Shimon-Hophy, Ramy R. Avtalion.** *Chronic Stress Down Regulate IFNγ in Cytotoxic Cells of the Common Carp (Cyprinus Carpio). Archives of Immunology and Allergy. 2018; 1(2): 54-66.*