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T-cells and CD45-cells discovery in the central nervous system of healthy and nodavirus-infected teleost fish *Dicentrarchus labrax*

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ABSTRACT

To achieve insights in antiviral immune defense of the central nervous system (CNS), we investigated T cells and CD45 cells in the marine fish model *Dicentrarchus labrax* infected with the CNS-tropic virus betanodavirus. By employing markers for pan-T cells (mAb DLT15) and CD45-cells (mAb DLT22) in immunofluorescence (IIF) of leukocytes from brain, we obtained 3.7 ± 2.3 % of T cells and 7.3 ± 3.2 % of CD45⁺ cells. Both IIF and immunoelectron microscopy confirmed a leukocyte/glia morphology for the immunoreactive cells. Quantitative immunohistochemistry (qIHC) of brain/eye sections showed 1.9 ± 0.8 % of T⁺ cells and 2 ± 0.9 % of CD45⁺ cells in the brain, and 3.6 ± 1.9 % and 4.1 ± 2.2 % in the eye, respectively. After *in vivo* RGNNV infection the number of T cells/CD45⁺ leukocytes in the brain increased to 8.3 ± 2.1 % and 11.6 ± 4.4 % (by IIF), and 26.1 ± 3.4 % and 45.6 ± 5.9 % (by qIHC), respectively. In the eye we counted after infection 8.5 ± 4.4 % of T cells and 10.2 ± 5.8 % of CD45 cells. Gene transcription analysis of brain mRNA revealed a strong increase of gene transcripts coding for: antiviral proteins Mx and ISG-12; T-cell related CD3 ϵ/δ , TcR β , CD4, CD8 α , CD45; and for immuno-modulatory cytokines TNF α , IL-2, IL-10. A *RAG-1* gene product was also present and upregulated, suggesting somatic recombination in the fish brain. Similar transcription data were obtained in the eye, albeit with differences. Our findings provide first evidence for a recruitment and involvement of T cells and CD45⁺ leukocytes in the fish eye-brain axis during antiviral responses and suggest similarities in the CNS immune defense across evolutionary distant vertebrates.

1. Introduction

The central nervous system (CNS) of vertebrates has been considered for decades an immune-privileged tissue not having exchanges of cells with the periphery, due to the presence of a blood-brain barrier (BBB). However, evidence accumulated that: i) the mammalian brain and the immune system are tightly intertwined [1], ii) CNS harbors resident leukocytes and iii) leukocytes can migrate from the blood via the BBB to CNS during pathogenic conditions [2,3]. The importance of CNS-associated immune activities has been recently shown in mice, where an eye-brain crosstalk during nervous viral infection has been

discovered [4]. Other evidences showed that mammalian T cells can enter the CNS [5] and cross the BBB during immunosurveillance and inflammation [6] in a complex interplay with cerebrospinal fluid (CSF) and brain interstitial fluid [3]. Both B and T lymphocytes are present in the CNS, and the human brain contains a few IgM-B cells and a much higher number of T cells [7]. The population of T cells includes both TcR $\alpha\beta/\gamma\delta$ phenotypes [8,9] with a slight outnumbering of the CD8⁺ subpopulation with respect to CD4⁺ in healthy mammalian brain [10]. The observed CD8⁺ presence can be likely due to the sensitivity of CNS towards viral infections, where cytotoxic T cells play a major defense role [11–13]. CD4⁺ T cells are also present in measurable amounts either as memory [10] and regulatory cells, supposed to constrain

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Abbreviations

ANOVA	Analysis of variance
BBB	blood-brain barrier
CNS	central nervous system
DAB	3,3'-diaminobenzidine
DIC	differential interference contrast
FCM	flow cytometry
FS/SS	forward scatter/side scatter
HBSS	Hank's balanced salt solution
IEM	immunoelectron microscopy
IIF	immunofluorescence
IHC	immunohistochemistry
MGG	May-Grünwald Giemsa
qPCR	quantitative polymerase-chain reaction
TCID	tissue culture infection dose
TRIS	tris(hydroxymethyl)aminomethane
RAG-1	recombinase-activating gene 1
SD	standard deviation

autoimmunity and excessive T cell activation [14].

A common feature of leukocytes is the CD45 surface molecule, that is differentially expressed in five isoforms in leukocyte subpopulations. In mammalian CNS the microglia, macrophages and the vast majority of lymphocytes have been shown to express CD45RB and CD45RO isoforms in both control and virus-induced encephalitis [15]. Interestingly, an increase in CD45RO⁺-T cells and macrophages in virus-infected CNS was observed, attributable to novel leukocyte infiltrates from the periphery. Overall, from the available knowledge it is evident the importance and the involvement of T cells and CD45⁺ leukocytes in cellular antiviral defense of mammalian CNS against viral infections and in neurodegenerative diseases [16]. Due to the similarities present in the anatomy and physiology of the immune system in the diverse vertebrates classes, teleost (bony) fishes are a good experimental model to investigate the evolutionary traits of immune defenses, as can be easily argued by the vast use of the zebrafish model. In this study, we utilized the Mediterranean sea bass (*Dicentrarchus labrax*) as a marine fish model, known for its susceptibility to the neurotrophic pathogenic virus betanodavirus. Leveraging specific markers previously identified for sea bass [17] and building upon prior knowledge of systemic immune defenses against betanodavirus [18], we conducted the first investigation in a fish into the effects of *in vivo* betanodavirus (RGNNV strain) infection on the abundance of sea bass CNS-associated T and CD45 cells. Additionally, we examined the transcription levels of master genes involved in immune responses. The findings suggest evolutionary similarities between teleost fish and mammals in certain CNS-related immune activities in response to viral infection, indicating the emergence of neuro-immunological activities dating back three hundred million years.

2. Materials and methods

2.1. Cell culture conditions and virus propagation

The viral isolate SpDIJAusc965.09 (RGNNV) [19] was spread on E11 cells [20] at 25 °C with Leibovitz (L-15) medium (Gibco) supplemented with 2 % fetal bovine serum (FBS, Gibco), 100 units/mL penicillin, and 10 mg/mL streptomycin (Sigma-Aldrich) until fully cytopathic effect (CPE) appearance. Supernatants were collected, centrifuged at 5000×g for 10 min at 4 °C, and the resulting viral suspension was titrated following the 50 % tissue culture infective dose method (TCID₅₀) (Reed and Muench, 1938) and stored at −80 °C until use.

2.2. Fish and RGNNV challenge

One hundred sea bass (*Dicentrarchus labrax*) juveniles (20 g, average weight), provided by CARMAR Cultivos Marinos (Almeria, Spain), were stabled for a viral challenge at the CEMSAC research centre of the University of Malaga (Malaga, Spain; Spanish Operational Code REGA ES290670002043). Animals were acclimated to experimental conditions for 2 weeks in a re-circulating system. Fish were maintained in 500-L tanks with aeration under stable temperature (23–25 °C) and salinity (35–37 g/L) conditions. The photoperiod was 12 h light: 12 h dark, and animals were fed (2 % biomass) during the experiment with a commercial pellet diet (Skretting, Spain). The absence of betanodavirus carriers was tested as already described [21,22].

For the RGNNV challenge, fish were randomly distributed into two tanks (control and RGNNV inoculated fish, N = 50 per group). RGNNV infection was carried out by intramuscular injection (i.m.) at a dose of 5×10^5 TCID₅₀/g fish. Animals from the control uninfected group were inoculated with serum-free L-15 medium. Five animals per experimental condition were killed by anaesthetic overdose with ethyl 3-aminobenzoate methanesulfonate (MS-222, Sigma) at 10 µg/mL at 3- and 6-days post-infection (dpi). Brain, eyes and muscle were aseptically collected and individually processed as indicated for histology, immunofluorescence (IIF), immunohistochemistry (IHC), immunoelectron microscopy (IEM) and qPCR analysis.

2.3. Cells and immunofluorescence

All the solutions employed were at the sea bass osmolarity of 350 mOsm/kg. Brains from 7 animals per group were removed, placed in cold HBSS and carefully cleaned of blood/blood vessels, then finely minced with a scalpel in a Petri dish and the tissue pushed sequentially through a 100 µm and a 40 µm nylon mesh using a syringe pestle and 10 mL/g of cold HBSS. The homogenate was centrifuged at 400×g at 4 °C, the pellet resuspended in 16 mL of cold HBSS and loaded over layers of Percoll in HBSS at densities of 1.02 and 1.04 g/cm³. The tubes were centrifuged at 550×g for 30 min with minimal acceleration and no brake. Cells at the 1.02/1.04 interface were collected, washed in HBSS at 400×g at 4 °C. Cells were counted in a hemocytometer and immediately employed for the analyses.

For IIF, 2.5×10^5 cells/tube were incubated on ice for 90 min with 2 mL of the mAbs DLT15 (IgG3) and DLT22 (IgG1) as undiluted culture supernatants, the cells were then washed as above and incubated for 30 min with a 1:250 dilution of a fluorescence-conjugated anti mouse IgG secondary antibody (AlexaFluor 594, Thermofisher), washed again as above, resuspended in 1 mL of PBS containing 0.2 % paraformaldehyde, and analyzed by flow cytometry (FCM) with an Epics XL-200 flow cytometer (Beckman Coulter). Same cell preparations were also observed and recorded with an optical microscope (Zeiss Axioscope) equipped with fluorescence.

2.4. Immunohistochemistry

Whole brain samples were collected from healthy sea bass adults (N = 3, aged 2 years), while both brain and eyes from control (uninfected, L-15) and RGNNV inoculated juvenile fish, at 3 and 6 dpi (N = 3 per experimental group, mean weight 18 g, 3 months-old). After removal, the samples were promptly transferred to ice-cold Bouin's fixative and incubated overnight before being stored in 80 % ethanol at 4 °C until further processing. Following the gradual dehydration by sequential ethanol-toluene washes, samples were embedded in paraplast plus medium (Sigma-Aldrich) and serial cross sections, 7 µm in thickness, of eye, forebrain, midbrain and hindbrain regions were obtained using a rotary microtome, collected on poly-L-lysine coated slides, and air-dried overnight at 37 °C. Some sections were stained with May-Grünwald Giemsa (MGG) staining (Pappenheim's panoptic method), for general histology determination, while others were processed for IHC performed

by ABC-peroxidase method, as previously described [23]. The mAbs DLT22 and DLT15, diluted 1:10 with PBS (0.1 M, pH 7.3) containing 5 % of seabass normal serum and horse normal serum (PSH) (Sigma-Aldrich), were employed for CD45 and T cells detection, respectively, in unchallenged adults and in both uninfected and infected (RGNNV) specimens. In each specimen, multiple sets of consecutive sections were differentially immunostained with the antibody mentioned above. Thereafter, sections were incubated with biotinylated horse anti-mouse IgG serum (Vector Labs, USA), as a secondary antibody, followed by incubation with avidin biotinylated peroxidase complex (ABC, Vectastain Elite, Vector Labs, USA). The staining was performed with 3, 3'-Diaminobenzidine (DAB) Enhanced Liquid Substrate System tetrahydrochloride (Sigma-Aldrich, USA). As negative control, the PSH replaced the primary antibodies. Distinct brain regions including the telencephalon (TEL), optic tectum (OT), cerebellum (CER) and retina were analyzed and the cell immunoreactivity was assessed and quantified by evaluating the number of positive cells in $10^5 \mu\text{m}^2$ (area). The sections were examined under a Zeiss Axioskop 2 plus microscope equipped with the Axiocam MRC camera and the Zeiss Zen 3.0 (blue edition) software (Carl Zeiss, Germany). Adobe Photoshop CC (Adobe Inc. Version 2021, Software) was employed for image contrast and brightness adjustment. For quantitative analysis (qIHC) of brain samples, nine non-consecutive sections per brain region were analyzed for each replicate. In the case of qIHC analysis of eye samples, six non-consecutive sections of the retina were examined instead for each biological replicate. The ImageJ software (version 1.54f, NIH, USA) was employed for cell counting. GraphPad Prism 8 statistical package was used for data analysis. Numerical data were expressed as the mean $\pm$ SD. One-way ANOVA followed by Tukey's post hoc test were used to determine differences among groups and the level for accepted statistical significance was $p < 0.05$.

2.5. Immunoelectron microscopy (IEM): post-embedding immunogold

The IEM investigation was performed on the brain region of the OT dissected along the brain dorsal-ventral axis of healthy sea bass juveniles ($N = 3$). Fresh tissues were quickly fixed in a mixture of 4 % paraformaldehyde (PFA) and 0.02 % glutaraldehyde in 0.1 M phosphate buffer (PB), pH 7.4, for 2 h at 4 °C, then rinsed three times with 0.1 M PB and incubated in a 0.2 M sucrose solution, overnight at 4 °C. Subsequently, samples were dehydrated in a graded ethanol series and embedded in LR White resin. The resin was polymerised in tightly capped gelatine capsules for 24 h at 50 °C. Ultrathin sections (80 nm) were obtained using a Reichert Ultracut ultramicrotome with a diamond knife and collected on nickel grids.

For immunogold staining (IGS), ultrathin sections were processed according to a two-step blocking procedure using Glycine 0.05 M in PBS for 15 min first, and then Aurion blocking solution for 30 min. After two sequential washes in the TRIS-HCl buffer, the sections were incubated with mAbs DLT15 or DLT22 (undiluted culture supernatant) overnight at 4 °C in a moist chamber. Then, after washes in TRIS-HCl, sections were incubated with the gold-conjugated goat anti-mouse antibody (British BioCell International, UK, gold particles 20 nm in diameter), diluted 1:20 in TRIS buffer (0.02 M, pH 8.2) for 2 h and then washed in PBS. Sections were subsequently stained with uranyl acetate and observed with a Jeol JEM EX II transmission electron microscope at 100 kV. Primary antibodies were omitted in control sections. Micrographs were acquired with an Olympus SIS VELETA CCD camera and ITEM software.

2.6. Gene expression analysis by real-time PCR

After bleeding the animals, brain and eye samples were surgically removed from both control (uninfected, L-15) and infected (RGNNV) sea bass specimens ($N = 3$ per experimental group) at 3 dpi and 6 dpi, and three biological replicates were used for each experimental condition.

Total RNA was extracted from the whole collected organs, using TRI Reagent (Sigma-Aldrich) according to the manufacturer's instructions, resuspended in molecular grade water and used for the qPCR assay. The purity and concentration of each sample were validated by spectrophotometric reading (Agilent Epoch 2, USA) and the integrity of 28S and 18S rRNA was evaluated via electrophoresis on a 1 % agarose gel stained with GelRed nucleic acid stain (Biotium Inc.). The cDNA was synthesized from 2 μg of RNA using the Tetro cDNA Synthesis Kit (Bioline), resuspended in molecular grade water and used in a dilution of 1:20 for qPCR. The absence of DNA contamination was assessed by amplifying the cDNA with β -actin primers that bracketed an intron (RV: 5'-CTCCTTAATGTACGCACGATTTC-3'; FW: 5'-ATCGTGGGGCGCCC-CAGGCACC-3'). Sixteen different primer pairs, specific for sea bass, were used for gene expression analysis as reported in [Supplementary Table 1](#). The qPCR was performed using Brilliant II SYBR green master mix (Sigma), with ROX as internal passive reference dye, on a Mx3000P Real Time PCR machine (Agilent Technologies, Inc., USA) and the cycling conditions were as follows:

initial denaturation at 95 °C for 10 min, followed by 36 cycles of denaturation at 95 °C for 30 s, annealing at 52 °C for 45 s, and extension at 72 °C for 45 s. Fluorescence data were collected at the end of the extension step of each cycle (endpoint method). The specificity of amplification was assessed by evaluating the melt curve following the qPCR cycling, which gave rise to a single distinct peak for each primer pair utilized. Samples in triplicate were employed for quantitative analysis. Relative gene expression was determined via the Livak method with Pfaffl correction for the efficiencies and using 18S ribosomal RNA as the housekeeping gene (normalizer target gene). Genes with the lowest expression in muscle tissue were employed as internal calibrators (*IL-1 β* , *CD4*, *RAG-1*). The results were graphically represented as the normalized relative quantities (NRQ) $\pm$ standard error of the normalized relative quantity (NRQ SE) and the significant differences among gene expression values were established by one-way ANOVA followed by the Tukey's post hoc test ($p < 0.05$).

3. Results

3.1. Effect of RGNNV infection

The intramuscular administration of betanodavirus at 5×10^5 TCID₅₀/g fish, at the experimental endpoint of 6 dpi, induced starting of external symptoms like skin blackening and sickness behavior identified as swimming abnormalities (swirling), as already reported [24]. The presence of RGNNV transcripts in the brain of infected fish at 6 dpi was determined by RT-PCR and confirmed in >90 % of sampled fish (not shown).

Brain homogenization produced with our protocol a mean of $4.5 \pm 1.2 \times 10^6$ cells/g, as counted after density gradients, with a viability (Trypan blue) > 90 %. An overall morphology of obtained cells can be seen in [Supplementary Fig. 1](#). From the figure it can be observed cells with a size of cs. 10 μm , likely corresponding to macrophage-like cells ([Fig. S1a](#)); cells with a lymphocytic morphology of 5–8 μm [[S1b-e](#)]; and dendritic-like cells, possibly representing microglia cells [[S1f](#)]. Other undefined material remained present in the cell preparation, likely corresponding to myelinated debris.

Incubation of live brain cells with mAbs DLT15 and DLT22 resulted in the staining of immunoreactive cells, as shown in [Fig. 1](#). Pan-T cells were positive to mAb DLT15 ([Fig. 1a, 1c, 1e](#)) showing a surface-associated membrane staining and a corresponding cell size in the range 6–10 μm attributable to lymphocytic cells. CD45⁺ cells showed a surface-associated membrane staining ([Fig. 1h, 1j, 1l](#)) with a leukocyte size in the range of either 6–10 μm or 10–12 μm , likely attributable to macrophages/glia cells.

Brain ultrathin sections were monitored by post-embedding IEM after incubation with mAbs and the obtained results can be seen in the lower panel of [Fig. 1](#). The mAb DLT15 produced a membrane staining of

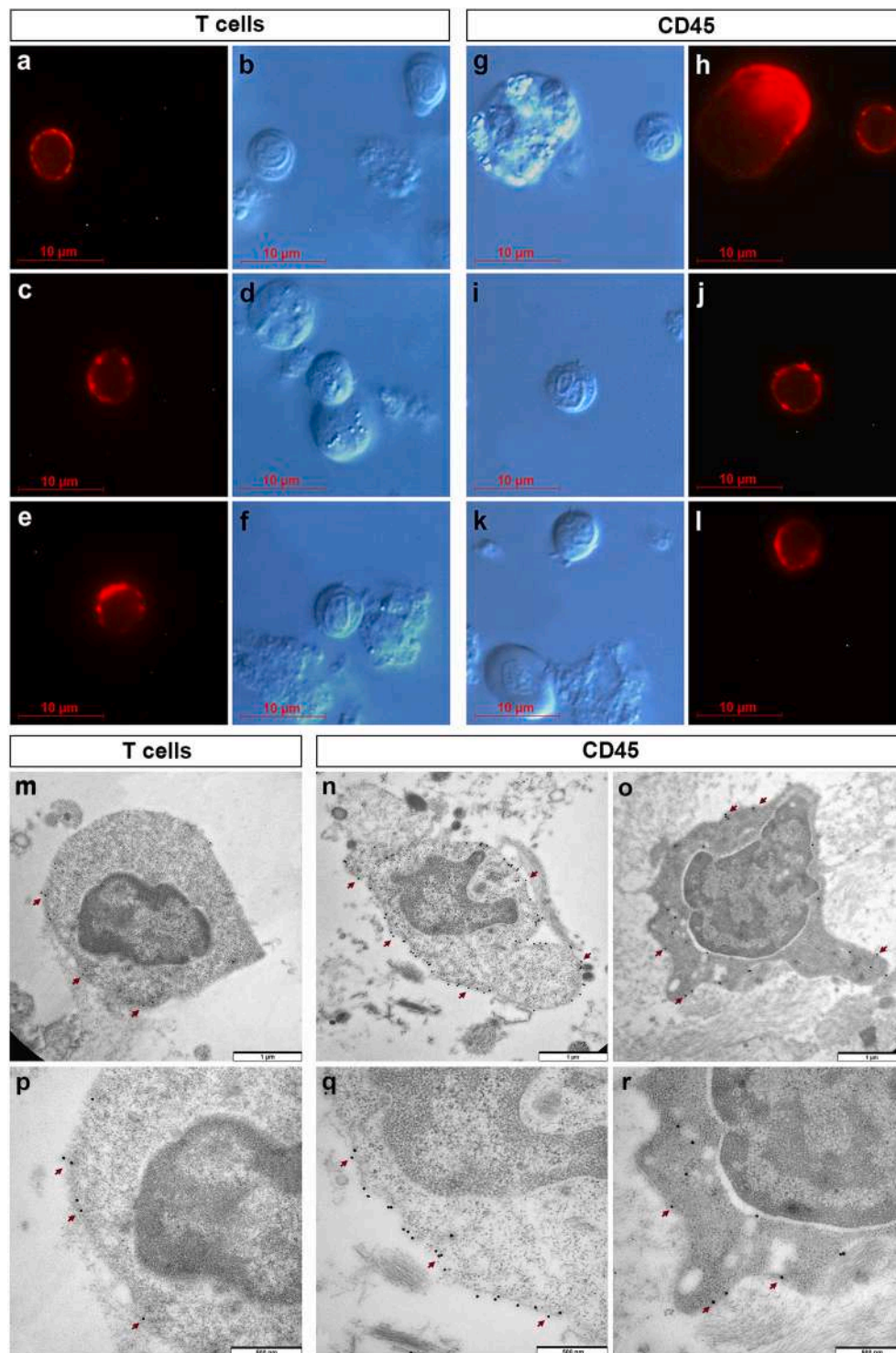


Fig. 1. Immunofluorescence and immunoelectron microscopy of cells from the brain. Sea bass brain derived leukocytes were stained with the mAbs DLT15 (T cells) and DLT22 (CD45 cells) and the antigen-antibody binding was revealed by indirect IIF (a, c, e, h, j, l). Both DLT15 (a, c, e) and DLT22 (h, j, l) recognized membrane antigens in cells with a size of 6–12 μm . The corresponding DIC images are shown in 1b, 1d, 1f, (DLT15) and 1g, 1i, 1k, (DLT22). Scale bars = 10 μm . Post-embedding immunogold of brain ultrathin sections is shown in 1m (DLT15) and 1n, 1o (DLT22). Higher magnifications are shown for both mAbs in (p) and (q–r), respectively. Gold nanoparticles are clearly detectable on the surface of immunoreactive cells. Scale bars = 1 μm (m, n, o) and 500 nm (p, q, r).

brain leukocytes having a morphology consistent with lymphocytes, displaying homogeneous cytoplasm and high nucleus/cytoplasm ratio, as can be appreciated in Fig. 1m and 1p. In agreement with other assays, the mAb DLT22 led to a higher number of membrane-associated gold particles in cells exhibiting macrophage/microglia morphology, with irregular shape and lobulated nuclei (1n, 1q, 1o, 1r).

Following the IIF the brain-associated leukocytes have been probed by FCM and the characteristic staining pattern in a typical experiment is shown in Fig. 2. The FS/SS morphology pattern shows a diffuse population of cells (Fig. 2a) that when probed with mAbs revealed the presence of a mean percentage of 3.7 ± 2.3 % of DLT15-positive pan-T cells (Fig. 2c) and of 7.3 ± 3.2 % of DLT22-immunoreactive CD45-cells

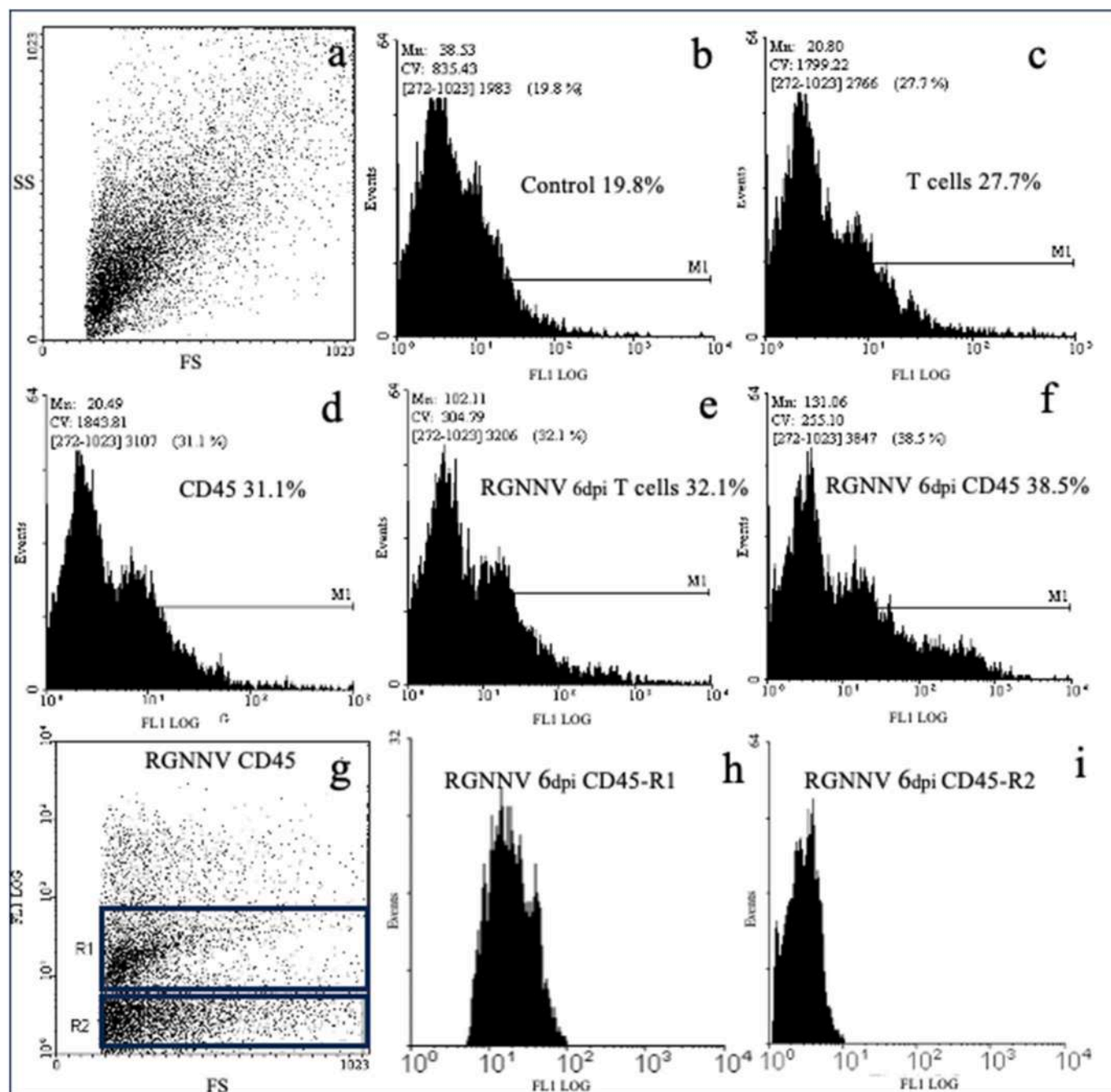


Fig. 2. Flow cytometry of cells from the brain. The FS/SS morphological pattern of unstimulated fish (control) is shown in (a) and the corresponding fluorescence is shown in (b) with a marker baseline line set at a 19.8 % value. The obtained percentages of T cells (DLT15⁺) are 27.7 % (c) and of CD45 cells (DLT22⁺) 31.1 % (d). The cytograms of brain cells from fish infected at 6 dpi and obtained positivity percentages are shown for T cells (e, 32.1 %) and CD45 cells (f, 38.5 %). In all cytograms the same set baseline value was maintained. For a further analysis, DLT22-stained brain cells were plotted FS/FL1 (g) and the two resulting populations gated as R1 and R2 (g) with the corresponding fluorescence shown in (h) and (i).

(Fig. 2d). After 6 dpi of nodavirus infections, the cytograms with the selected threshold showed an increase of pan-T cells (12.3 ± 2.1 %, Fig. 2e) and CD45-cells (18.6 ± 4.4 %, Fig. 2f). By analyzing the cell population positive to DLT22 (CD45-cells, Fig. 2g), the IIF positivity appears to be restricted to the R1 single population (Fig. 2h), as can be appreciated in the gate selection of Fig. 2g.

3.2. Immunohistochemistry shows the *in situ* presence of CNS-associated leukocytes

The conspicuous amount of data obtained by IHC on brain and eye

sections has been summarized in Figs. 3–6. The fish brain, like other vertebrates, contains the three regions indicated in upper Fig. 3, namely telencephalon (TEL), optic tectum (OT), cerebellum (CER) and these regions were employed to obtain sections from areas as shown in upper Fig. 3. Investigating an adult sea bass (ca. 2-years old) the histological staining of brain areas can be appreciated in Fig. 3a, 3b, 3c, displaying the typical organization and features as previously described in sea bass [25]. With respect to resident leukocyte staining, in TEL both T cells (Fig. 3d) and CD45-cells (Fig. 3g) were in a low number and mostly present along the median channel. The amount of T and CD45 cells was evident in the OT parenchyma and differentially distributed inside the

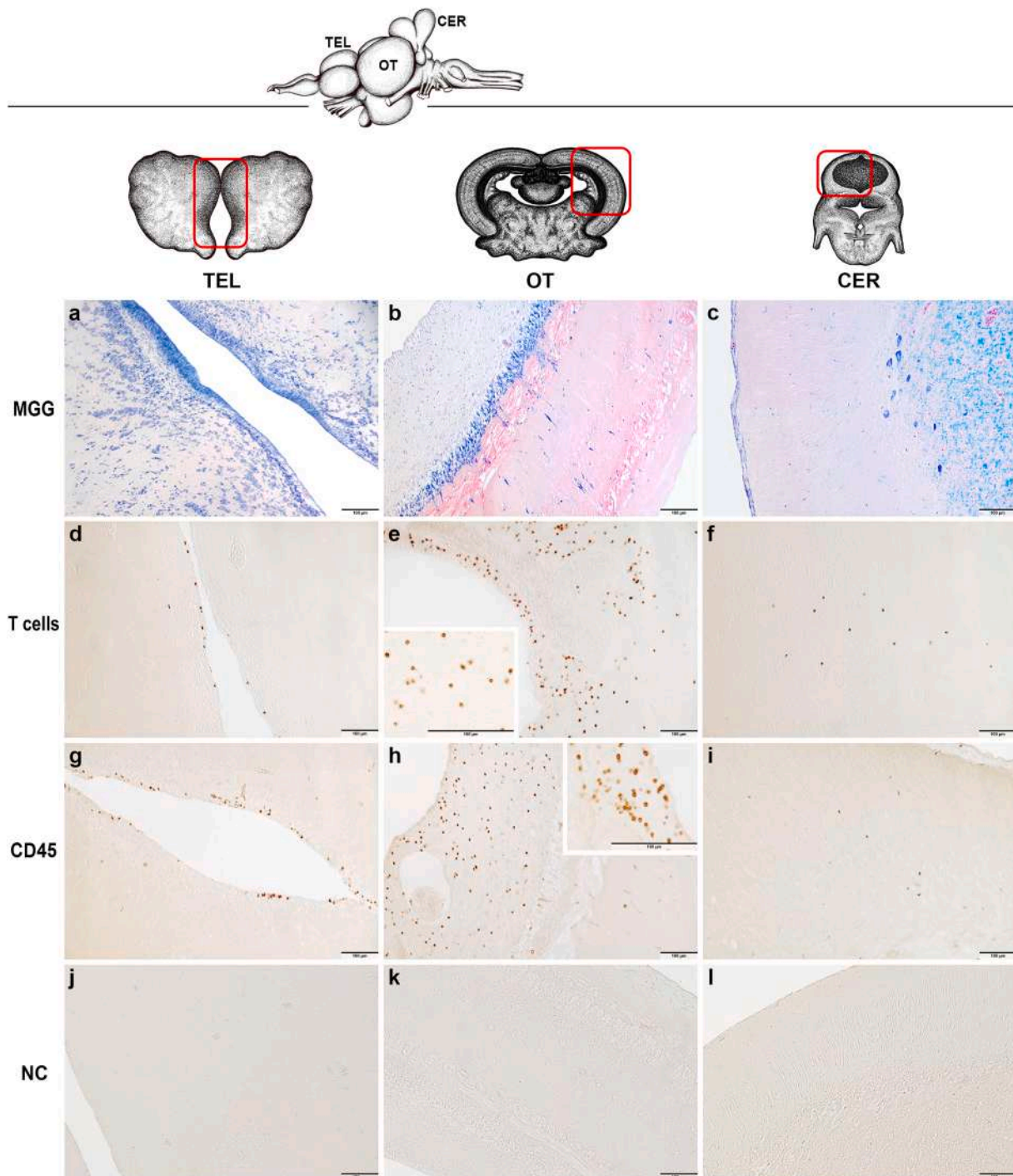


Fig. 3. Immunohistochemistry of brain regions from uninfected adult fish. A simplified draw of the sea bass brain and sagittal sections of TEL, OT, CER are shown from left to right. May-Grünwald Giemsa (MGG) stained sections are shown for TEL (a), OT (b) and CER (c). The sections have been incubated with mAbs and obtained immunostaining is shown for T cells in TEL (d), OT (e), CER (f) and for CD45 cells in TEL (g), OT (h), CER (i). Negative controls are shown for TEL (j), OT (k) and CER (l). Scale bars = 100 μ m.

OT area (Fig. 3e and 3h, respectively). In healthy fish also the CER contains a low number of resident T (Fig. 3f) and CD45 cells (Fig. 3i).

The presence of pan-T cells and CD45-cells was also investigated by IHC in the brains and eye of sea bass juveniles infected i.m. with RGNNV and the results are shown in Figs. 4-6 at 3 dpi and 6 dpi. To be remembered, 6 days is the time when fish display clear behavior sickness. With respect to control sections obtained from uninfected fish injected with L-15 medium alone (the L-15 is the culture medium of virus-producing cells) and displayed, for the brain, in Figs. 4a-4fa (upper

panel), at 3 dpi a slight increase in - DLT15⁺ was observed in TEL (Fig. 4g), and in CD45⁺ cells in both TEL and OT (Figs. 4j, 4k). Only rare T cells and CD45-cells were instead found in CER (Figs. 4i, 4l).

At 6 dpi (lower panel) a dramatic increase of immunoreactive cells was found in TEL, OT and CER for both T cells (Figs. 4m-4o) and CD45-cells (Figs. 4p-4r), with an appreciable higher number for the latter. The positive cells were distributed throughout the brain parenchyma without any evident accumulation/depletion areas.

Quantitative data on the number of immunoreactive cells present in

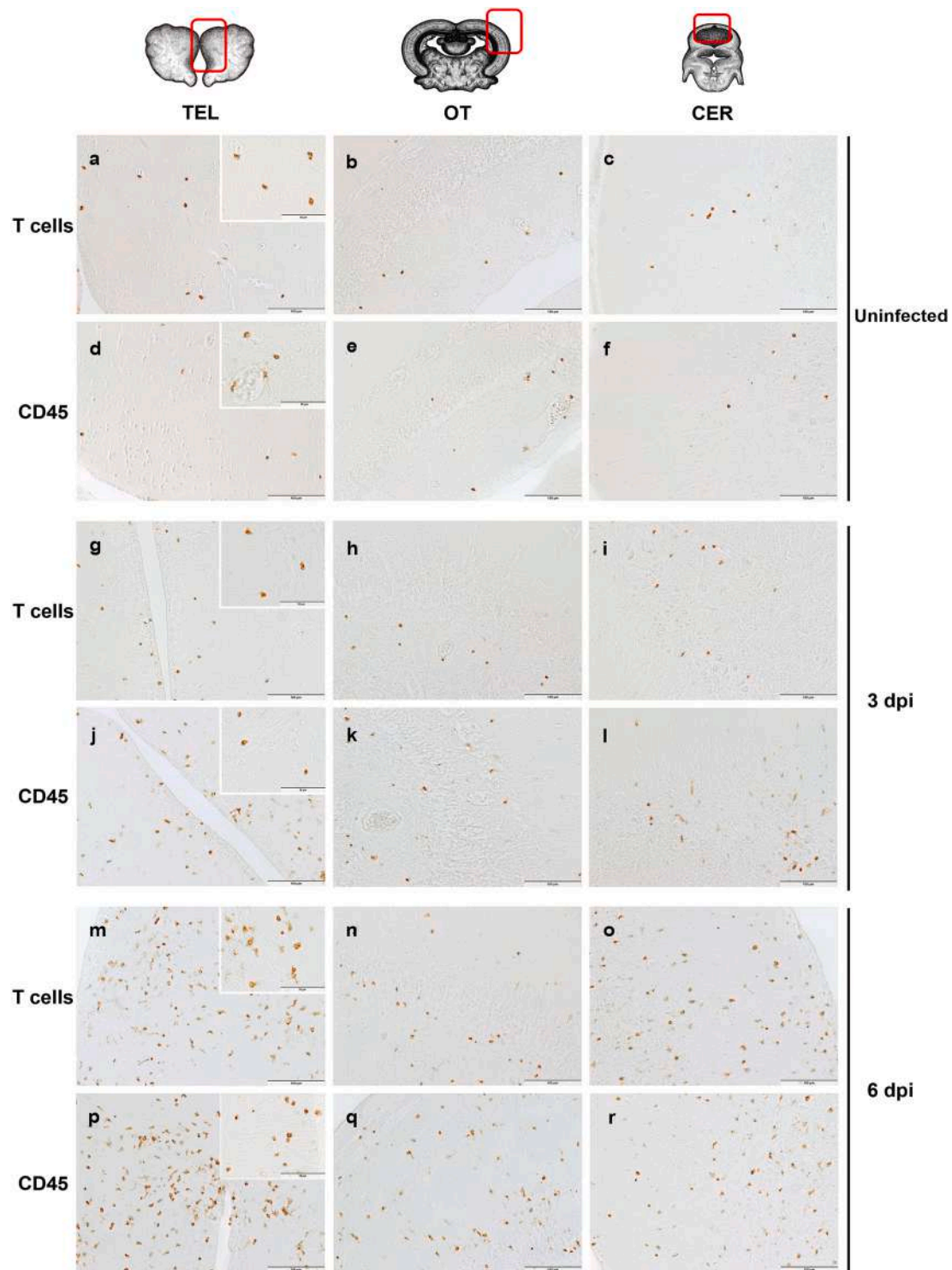


Fig. 4. Immunohistochemistry of brain regions from RGNNV-infected fish. T cells (DLT15⁺) and CD45 cells (DLT22⁺) from brain regions (TEL, OT and CER) of uninfected fish (injected with L-15) are shown in (a, b, c) and (d, e, f), respectively. A higher magnification of immunoreactive cells is shown in insets (a, d). The immunostaining of TEL, OT, CER, obtained from RGNNV-infected fish is shown at 3 dpi (g–l) and 6 dpi (m–r). At 3dpi T cells and CD45 cells are shown in 4g–4i and 4j–4l, while at 6 dpi in 4m–4o and 4p–4r, respectively. A higher magnification of immunoreactive cells is shown in insets (g, j and m,p). Scale bars = 50 and 100 μm are indicated.

the considered sections from the brain and evaluated by computer-assisted image analysis are given in Fig. 5(a–d) as the mean $\pm$ SD number of positive cells in 100.000 μm^2 .

The upper panel (Fig. 5a) shows pan-T cells immunoreactive to the mAb DLT15 as they were counted in TEL, OT and CER in uninfected fish,

oTfr injected with L-15 (black bars), and in fish infected at 3 dpi (empty bars) and 6 dpi (gray bars). An increase of T cells in TEL only can be appreciated at 3 dpi, whereas a strong rise was revealed in the three regions at 6 dpi. With respect to the control fish, at 6 dpi the increase of T cells in the TEL was from 2.4 ± 1.1 to 30.3 ± 10.8 ; in OT from 1.2 ± 1.4

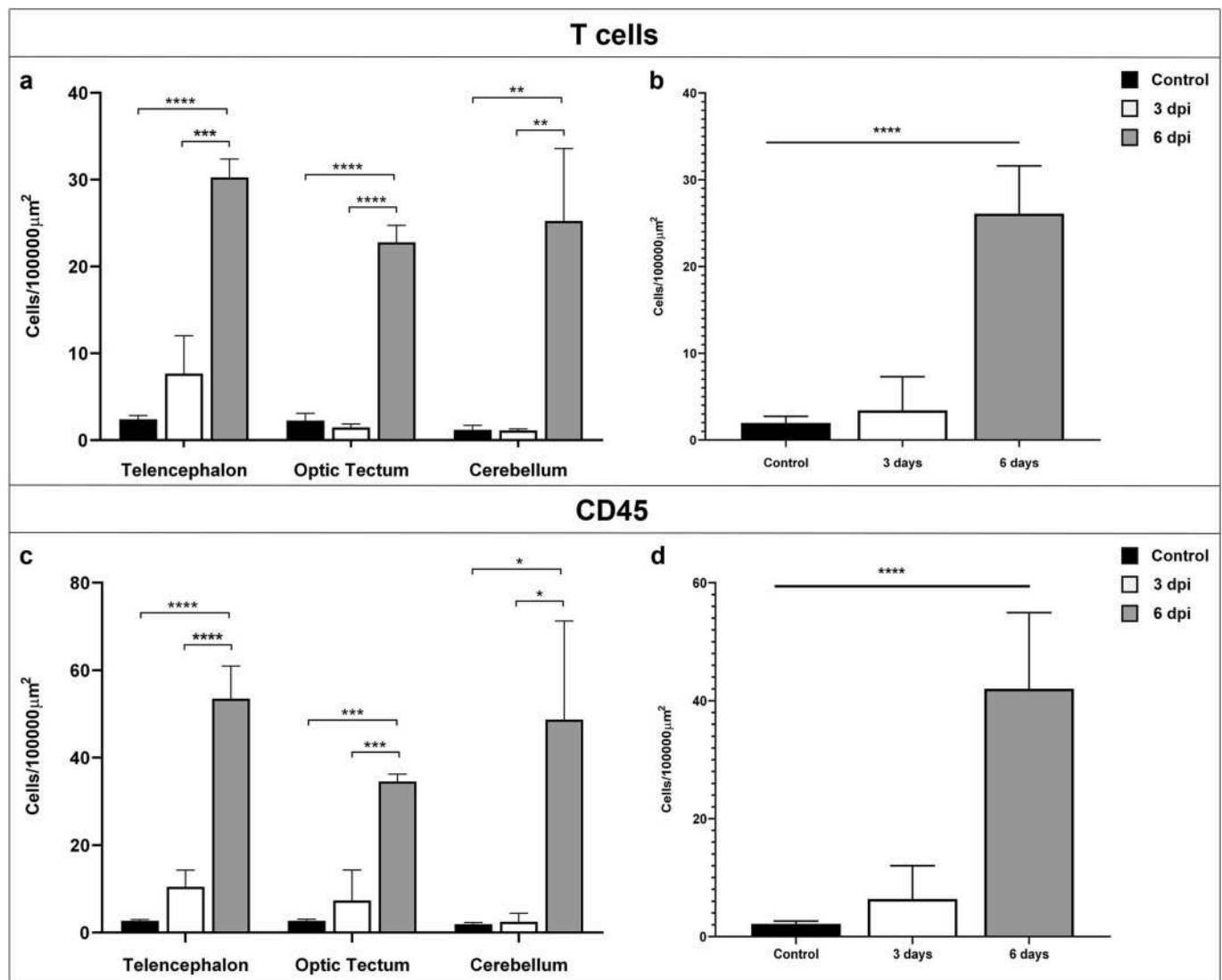


Fig. 5. Quantitative immunohistochemistry data from brain regions. Positive cells in 100,000 μm^2 from brain regions (TEL, OT, CER) and the whole brain in controls and fish infected at 3 dpi and 6 dpi are shown in 5a, 5b for T cells and 5c, 5d for CD45 cells. One-way ANOVA followed by Tukey's post hoc test were used to determine differences among groups and the level for accepted statistical significance between samples and groups was $p < 0.05$. The significance values are indicated by asterisks (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$).

to 22.8 ± 4.7 ; in CER from 1.2 ± 0.5 to 25.2 ± 6.3 . By considering the brain as a whole the qIHC data for pan-T cells were 1.9 ± 0.1 in the control group, 3.4 ± 1.3 at 3dpi, and 26.1 ± 3.4 at 6 dpi (Fig. 5b). A similar increase was measured for CD45-cells in brain regions (Fig. 5c) and as a whole resulted 2 ± 0.8 in controls, 5.9 ± 4.7 at 3 dpi and 42.3 ± 12.5 at 6 dpi (Fig. 5d).

Due to the pathogenesis of RGNNV, causing retinopathy, the analysis was extended to the eye and the results shown in Fig. 6. Histological staining with MGG showed the typical tissue organization of the retina (a) and IHC confirmed the retina as a site where leukocytes became present after RGNNV infection. In uninfected fish, no appreciable positivity for both mAbs was detected (Fig. 6b), whereas an evident colonization of T (Fig. 6c) and CD45⁺ cells (Fig. 6d) was clearly seen at 6 dpi. Quantitative analysis of IHC-immunoreactive cells in eye was performed, as well as for the brain, and results (Fig. 6 lower panel) showed in the retina that at 3 dpi there was not a measurable difference between uninfected and infected fish, whereas at 6 dpi T and CD45⁺ cells were 8.5 ± 4.4 % (Figs. 6e) and 10.2 ± 5.8 % (Fig. 6f), respectively.

3.3. Quantitative PCR for transcription of genes from brain and eyes

Whole brain and eyes were processed to obtain mRNAs, whose transcription levels have been evaluated by qPCR with the specific primers listed in Supplementary Table S1 and arbitrarily subdivided into three panels: i) T cell-related genes (*CD3 ϵ / δ* , *CD3 β* , *TcR γ* , *CD4*, *CD8 α*); ii) innate antiviral and proinflammatory genes (*Mx*, *ISG-12*, *IL-1 β* , *TNFA*); and iii) general acquired immunity-related genes (*IL-2*, *IFN- γ* , *RAG-1*, *CD45*). The gene expression profile, given with respect to a calibrator gene, is shown for each panel in Figs. 7–9 for the brain (a) and eye (b), respectively. In general, the results obtained by qPCR confirmed the involvement of T and CD45 cells in fish antiviral responses. More in detail, the genes from the group of T cell-related genes (Fig. 7a) resulted significantly upregulated in the brain at 3 and 6 dpi compared to control, with the exclusion of *TcR γ* . The infection-induced upregulation was evident at 3 dpi and it further increased at 6 dpi showing a higher level for *CD3 δ* , followed by *CD4*, *TcR β* , *CD8 α* and *CD3 ϵ* . The calibrator gene (arbitrary value 1) was *CD4* from skeletal muscle. In the eye (Fig. 7b) all the selected T cell-related genes did not present statistically relevant differences among groups. As expected, the expression of antiviral genes

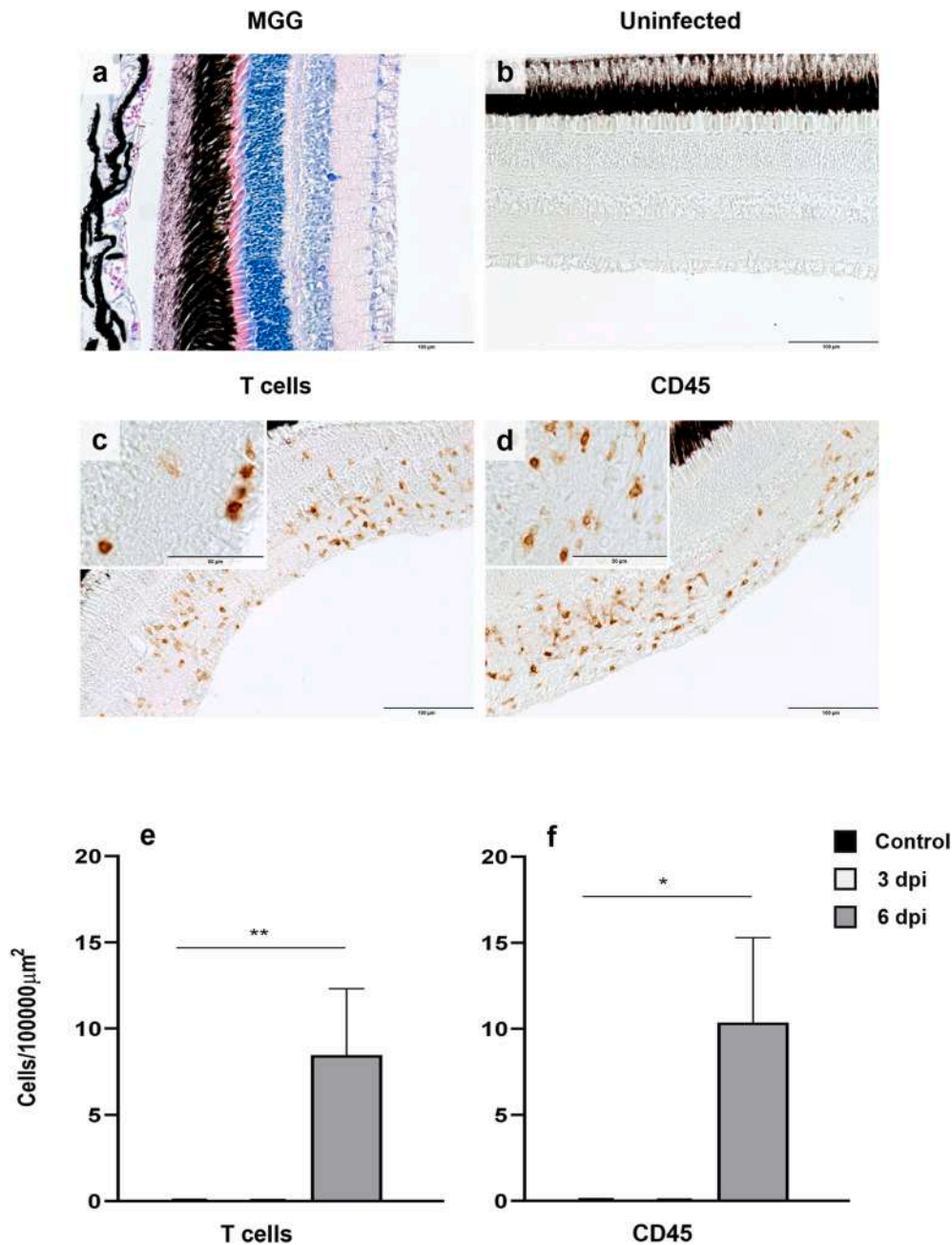


Fig. 6. Immunohistochemistry of eye tissues. Eye sections from infected fish (6 dpi) were stained with - May-Grünwald Giemsa (MGG), evidencing retinal layers (a), and mAbs DLT15 for T cells (c) and DLT22 for CD45 cells (d). Fish injected with L-15 (uninfected) did not reveal any staining (b). Higher magnification of immunoreactive cells can be seen in insets (c, d). Scale bars of 100 μm are shown. The immunoreactive cells in eye sections were expressed for T cells (e) and CD45 cells (f) as the number of positive cells/100.000 μm^2 . One-way ANOVA followed by Tukey's post hoc test were used to determine differences among groups and the level for accepted statistical significance between samples and groups was $p < 0.05$. The significance values are indicated by asterisks (* $p < 0.05$; ** $p < 0.01$).

Mx- and *ISG-12* (Fig. 8a) was potently and significantly upregulated in the brain after RGNNV infection, reaching approximately three orders of magnitude compared to the control group. Transcripts for the two proinflammatory cytokines *TNF α* and *IL-1 β* showed quite a different behavior, with the former displaying a significant expression increase and the latter remaining largely unchanged. Similar to the brain, the antiviral and proinflammatory genes were significantly upregulated in the eye at both 3 and 6 dpi, with the exclusion of *IL-1 β* , whose expression remained unchanged (Fig. 8b). For this gene group the calibrator gene (arbitrary value 1) was *IL-1 β* from skeletal muscle. In the third group named as general acquired immunity-related genes, all the selected gene transcripts from the brain were upregulated by virus infection (Fig. 9a).

In particular, *IL-2* and *INF γ* exhibited a stronger increase, followed by *CD45* and *IL-10*. Notably, the *RAG-1* gene, responsible for lymphocyte somatic recombination, was expressed in the brain and significantly upregulated in response to viral infection. By employing the third gene group in the eye (Fig. 9b), a significant increase in gene transcription for *IL-10* and *INF γ* was observed at 6 dpi. Conversely, a tendency towards upregulation after infection was recorded for *IL-2*, *RAG-1* and *CD45* genes, although the differences did not reach statistical significance. For this latter gene group the calibrator gene (arbitrary value 1) was *RAG-1* in skeletal muscle.

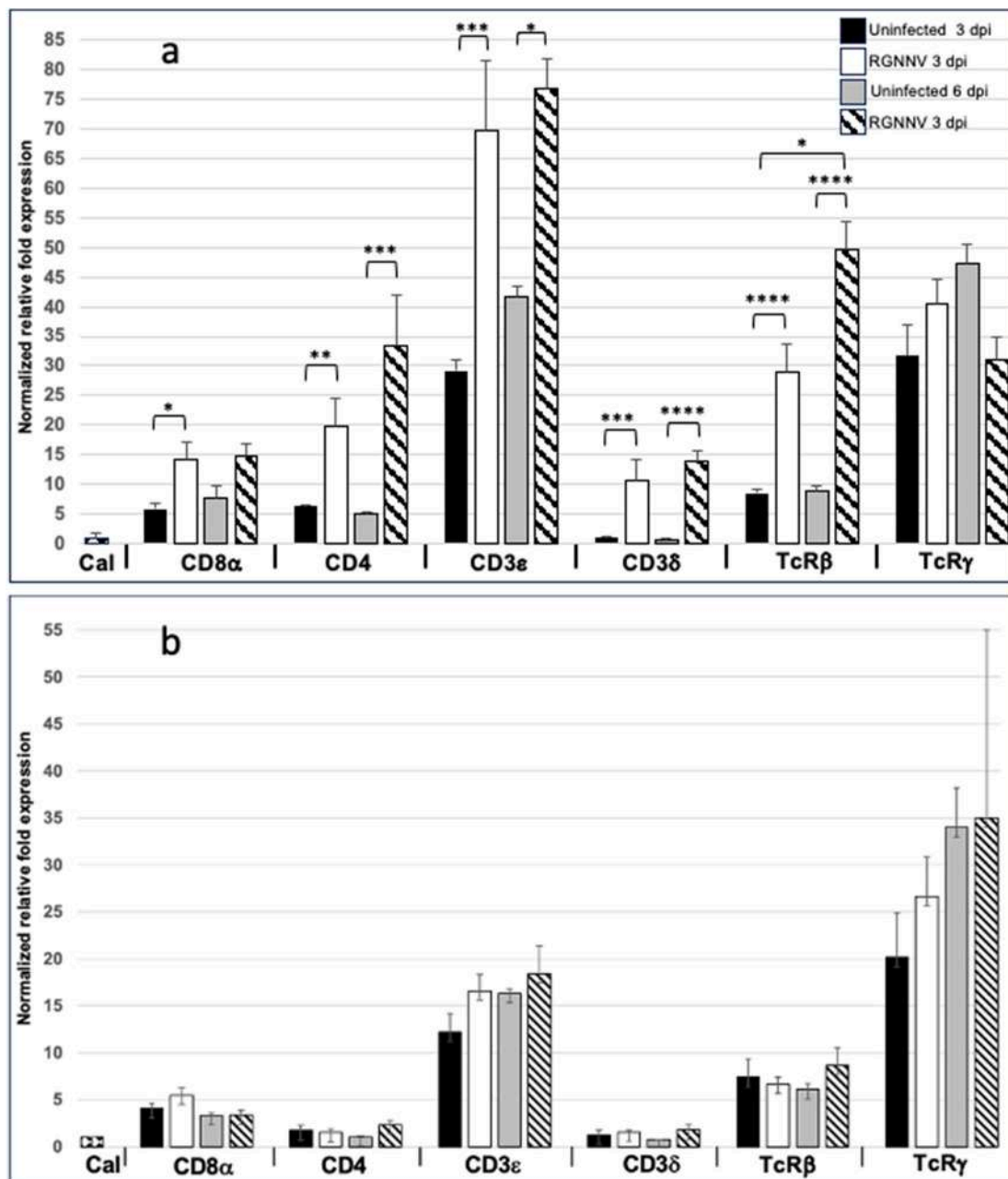


Fig. 7. Quantitative PCR of selected T cell-related genes from the whole brain and eyes. *CD8α*, *CD4*, *CD3ε*, *CD3δ*, *TcRβ* and *TcRγ* expression in the whole brain (a) and eyes (b). The mRNA levels were expressed as a ratio relative to 18S rRNA in the same samples after real-time PCR analysis using the tissue with the lowest *CD4* expression (muscle) as the calibrator. One-way ANOVA followed by Tukey's post hoc test were used to determine differences among groups and the level for accepted statistical significance between samples and groups was $p < 0.05$. The significance values are indicated by asterisks (* $p < 0.05$; ** $p < 0.01$).

4. Discussion

Viral infections of vertebrate CNS are the main cause of encephalitis, inducing brain parenchyma inflammation and subsequent recruitment of leukocytes from the periphery across the BBB. This process, poorly understood until some time ago [2], recently was shown to take place in mammals through a brain-associated lymphatic system [3]. Among the viruses causing CNS infection with consequent encephalitis and inflammation, it can be remembered the zika virus in humans [26], the neurotrophic mouse hepatitis virus (NMHV) and the herpes simplex-1 (HSV-1) in mice [27,28]. In these pathologies, among other defense mechanisms, it was postulated an important role in the brain for CD45-cells and T cells [29,30], with the presence of CD4/8-T cell subpopulations contributing to virus control [27,28]. To investigate the

evolutionary origin and conservation of CNS-associated immune responses across vertebrate classes, we selected a marine fish model, the Mediterranean sea bass. For this teleost, comprehensive knowledge on the infection of CNS by the neurotrophic retrovirus betanodavirus (*Nodaviridae*, RGNNV strain) inducing neuropathy and retinopathy is readily accessible. Previous studies in sea bass investigated some *in vivo* and *in vitro* leukocyte antiviral activities, the production of long-lasting specific antibodies and the effects of vaccination on immune related gene transcription [18,31–33]. These studies revealed that the sea bass is capable of mounting a clear systemic cellular and humoral response against RGNNV. Available knowledge also indicated that RGNNV pathology is restricted to young specimens, resulting in a potential complete loss of juvenile production in farming conditions, while older fish exhibit lower sensitivity. Aged sea bass are likely survivors to the

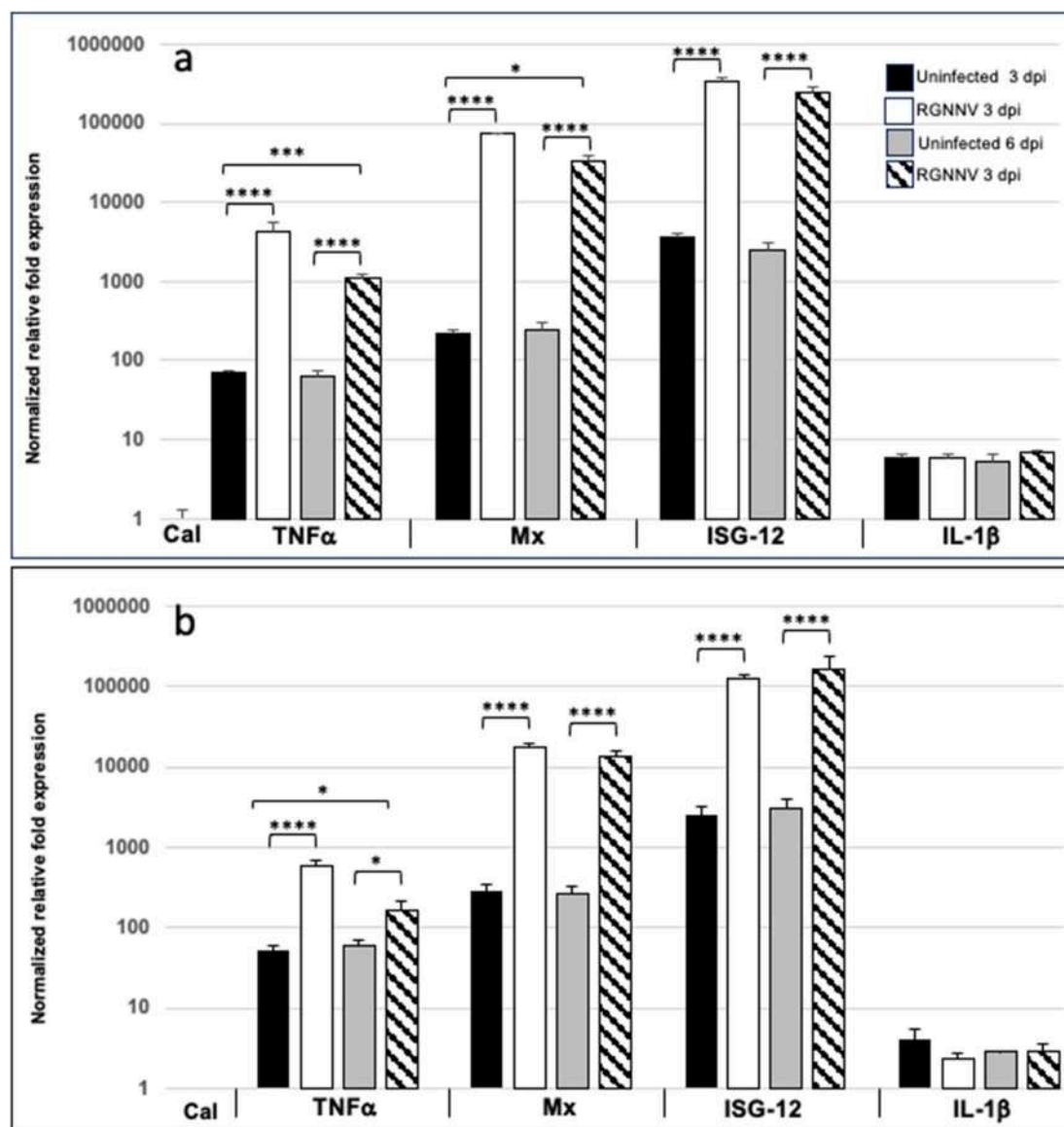


Fig. 8. Quantitative PCR of selected antiviral and proinflammatory genes from the brain and eye. *TNFα*, *Mx*, *ISG-12* and *IL-1β* expression in the whole brain (a) and eyes (b). mRNA levels were expressed as a ratio relative to 18S rRNA in the same samples after real-time PCR analysis using the tissue with the lowest *IL-1β* expression (muscle) as the calibrator. One-way ANOVA followed by Tukey's post hoc test were used to determine differences among groups and the level for accepted statistical significance between samples and groups was $p < 0.05$. The significance values are indicated by asterisks (* $p < 0.05$; ** $p < 0.01$).

infection and possibly protected, showing measurable specific IgM antibody titers against the virus [34]. By taking advantage of specific mAb markers for pan-T cells (DLT15) and CD45-cells (DLT22) which are available for sea bass, we investigated the presence of these cell populations in the brain of healthy fish and during a brain-specific viral infection. Previous studies performed by antigen sequencing and *in vitro* leukocyte assays suggested that the cells recognized by the DLT22 mAb are likely attributable to the CD45RO subpopulation [17]. A first step was the isolation of brain leukocytes and their analysis, as shown in Figs. 1–2 and Supplementary Fig. 1. By employing our protocol, the results showed that from the brain of sea bass juveniles (15–20 g), an average of $4.5 \pm 1.2 \times 10^6$ leukocytes/g brain can be obtained. When the live leukocytes were probed by IIF with mAbs DLT15 (pan-T cells) and DLT22 (CD45-cells), the resulting immunostaining resulted consistent with expected results. From Fig. 1 the mAb DLT15 revealed a membrane staining of cells having the size of lymphocytes (6–10 μ m) and the mAb DLT22 of cells in the range 6–12 μ m, attributable to lymphocytes and macrophages/glia cells. Importantly, other cells present

in the brain cell preparation (see Fig. 1) were not stained by the mAbs, thus confirming their specificity. A more detailed analysis by post-embedding immunogold labeling of DLT15 and DLT22 immunoreactive cells (Fig. 1) revealed intense and specific accumulation of gold nanoparticles in the plasma membrane of cells displaying ultrastructural features consistent with lymphocytes and macrophages/glia cells. The IIF analyses were monitored by FCM (Fig. 2) that supplied the percentages of immunoreactive cells in juveniles brain preparations, being 3.7 ± 2.3 % of positive pan-T cells and of 7.3 ± 3.2 % of CD45 cells. - To study in the fish model the effects of a CNS-specific viral infection, an *in vivo* intramuscular infection with betanodavirus (RGNNV strain) was performed for 3 and 6 days. The infection endpoint timing (6 dpi) was selected on the basis of when, commonly, the fish started to show clear sickness behavior symptoms like circle swimming, as already reported [24]. Indeed, as measured by IIF at 6 dpi the infection increased the number of brain-associated leukocytes to 8.3 ± 2.1 % for pan-T cells and 11.6 ± 4.4 % for CD45 cells. Interestingly, the cytogram of the CD45-positive cells obtained plotting FL1 versus FS revealed two

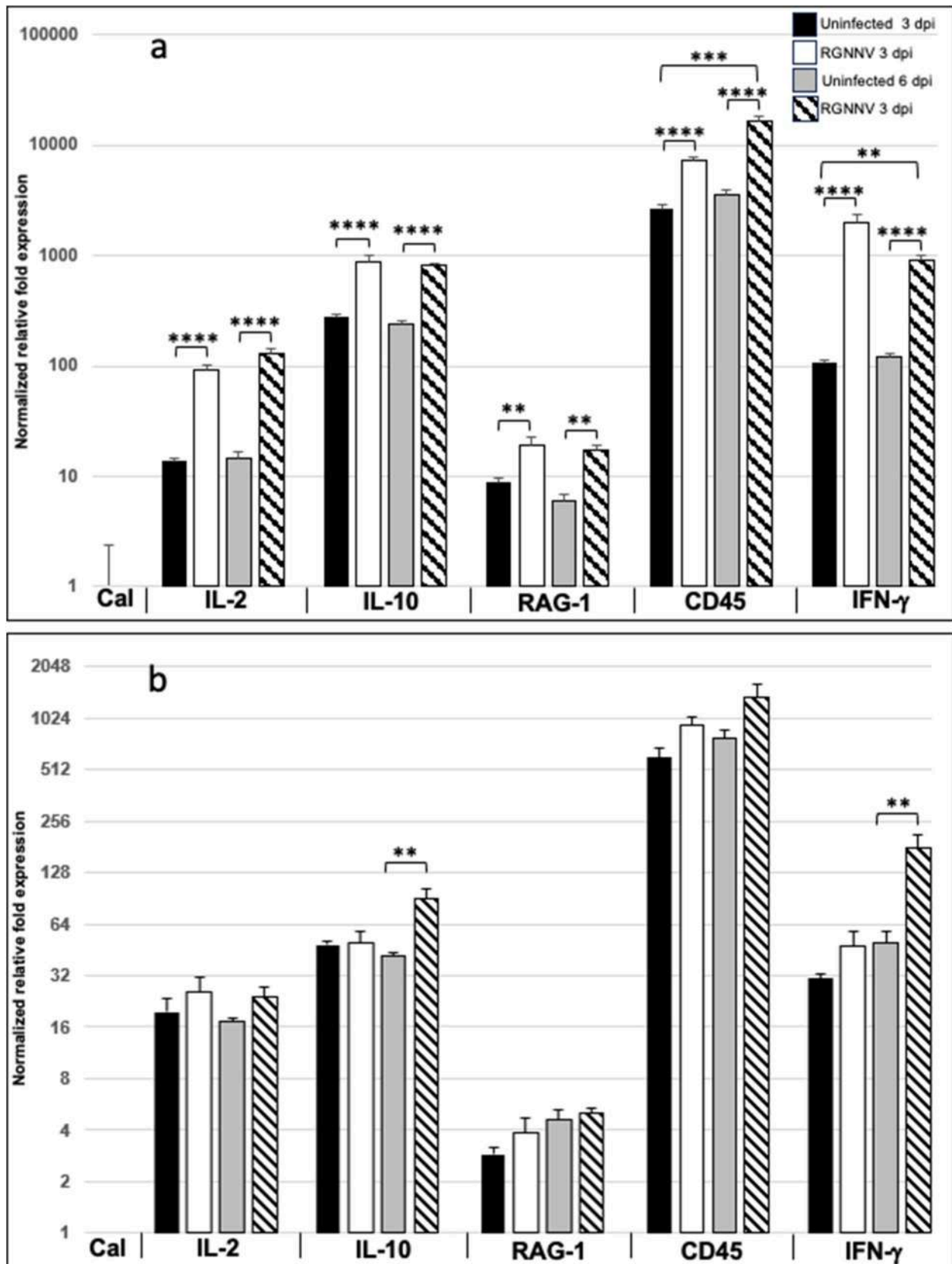


Fig. 9. Quantitative PCR of selected genes related to general acquired immunity from the whole brain and eyes. *IL-2*, *IL-10*, *RAG-1*, *CD45* and *IFN-γ* expression in the whole brain (a) and eye (b). mRNA levels were expressed as a ratio relative to 18S rRNA in the same samples after real-time PCR analysis using the tissue with the lowest *RAG-1* expression (muscle) as the calibrator. One-way ANOVA followed by Tukey's post hoc test were used to determine differences among groups and the level for accepted statistical significance between samples and groups was $p < 0.05$. The significance values are indicated by asterisks (* $p < 0.05$; ** $p < 0.01$).

distinct leukocyte populations, of which the one selected (R1, Fig. 2g and 2h) accounted for almost all the positivity, suggesting the recognition of a defined brain leukocyte population. However, we cannot exclude that the amount of positive cells measured by IIF and FCM might also contain some leukocytes coming from contaminant blood that may be present in the brain cell preparation despite the fish bleeding and careful cleaning of the tissue prior homogenization.

To better visualize immunoreactive cells in the brain parenchyma the following step was the *in situ* identification of immunoreactive leukocytes by IHC and quantitative IHC, with the results displayed in Fig. 3–5. The sea bass brain was probed in sagittal sections obtained from the three main areas of TEL, OT and CER, as indicated in Fig. 3. In adult healthy sea bass the presence of both pan-T cells and CD45 cells was evident in OT, whereas TEL and CER contained only a few of positive cells. It is particularly stimulating to observe the regional difference in the number of positive cells detected between the three brain areas and inside the OT stroma, possibly representing a site-specific tropism of brain leukocytes. As a comparison, the presence of resident T cells in the human brain was previously shown, where T cells represented a conspicuous part of CNS-associated immune cells [35], with a preponderance of the CD4⁺ phenotype. These cells are supposed to provide protection against neurotropic virus infection and reactivation [10,36]. Unfortunately, we cannot discriminate by IHC the T cell population of sea bass into CD4⁺/CD8⁺ subpopulations with specific cellular markers, and future work will be devoted to this. It is known that, in the healthy human brain, the microglia express CD45 [37] and thus our data in fish demonstrate the origin of CNS-associated CD45 cells early in jawed vertebrate history. Moreover, the CD45RO is a predominant CD45 isoform expressed in human control brain [37] and the results from our previous work suggested that CD45RO⁺ cells should also be a population recognized by the mAb DLT22 [17]. A difference was observed between the number of lymphocytes housed in the OT of healthy adult fish (2 years-old, Fig. 3) and in experimental young fish (Fig. 4). This discrepancy could be attributable, as in mammals, to a physiological numerical increase of brain T cells with aging [38]. However, there is no knowledge, at present, on age-dependent increase of T cells in the sea bass brain and this needs further investigation before to draw definitive comparison.

As for the IIF data, the *in vivo* infection of sea bass juveniles with RGNNV (see methods for details) resulted in a dramatic recruitment of leukocytes after 6 days in the brain parenchyma and in the eye, an anatomical extension of the CNS, as can be clearly seen in Fig. 4–6. With respect to the control fish, showing scarce positive cells (Fig. 4a–4f), after 3 days of infection a low number of T cells and CD45-cells can be observed in TEL (Fig. 4g, 4J), but at 6 dpi their amount in the parenchyma of TEL, OT and CER was impressive, as from Figs. 4m–4r. The great presence of T cells and CD45-cells in the moment (6 dpi) when fish show unequivocally sickness behavior symptoms of RGNNV suggests a deep involvement of these cell populations in antiviral immune activities of the CNS. The important role played by T cells and CD45-cells in virus-induced encephalitis has been already reported in mammals (see above for references) and, interestingly, in a previous work the peak of herpes virus infection in the mouse brain was detected at 6 dpi, similarly to sea bass, together with a T cell extravasation into the infected brain regions [39]. Similar considerations pertain to the eye, as it is also a common target for RGNNV infection. In the eye at 3 dpi, no differences were observed by IHC with respect to controls (data not shown), but at 6 dpi the presence of T cells and CD45 cells was conspicuous in the retina (Fig. 6c, 6d), with a higher number of CD45 cells. Recently, the involvement of antiviral immune responses in the brain and eye have been shown to take place in mammals and suggested the possibility of antigen delivery by ocular immunization in mice [4]. The qualitative IHC results have been flanked by quantitative evaluation of positive cells by computer-assisted image analysis with statistical significance (Figs. 5–6). The qIHC was evaluated for individual TEL, OT, CER areas and for the whole brain, and the results clearly suggested a detectable

increase of T cells and CD45 cells in all areas at 3 dpi, followed by a dramatic and significant rise at 6 dpi (Fig. 5a–c), achieving incremental values (for the whole brain) of ca. 13 times for T cells (Fig. 5b) and ca. 21 times for CD45-cells (Fig. 5d). To the best of our knowledge, these increment values represent the first quantitative *in situ* report on the presence of T cells and CD45-cells in a teleost brain after an experimental viral infection. The infection, at 6 dpi, resulted in appreciable vacuolization of the brain parenchyma, as a typical RGNNV effect (not shown). In order to verify whether the observed leukocyte presence in the healthy and infected brains and eye had a counterpart in gene transcription, we performed qPCR analysis with a set of specific genes we divided into bunches representative of cellular activities related to T cells, antiviral/proinflammatory and general acquired immunity. The results of qPCR, shown in Figs. 7–9 appear to be in strong agreement with the IHC results. In term of T cell activities, transcription of master genes coding for CD3 ϵ -chain, CD3 δ -chain, CD4, CD8 α -chain, TcR β -chain showed statistically increased expression levels, albeit to varying extents at 3 dpi and predominantly at 6 dpi, with the exclusion of TcR γ -chain that did not reach statistical significance. The increase in T cells measured by IIF/qIHC was flanked by the increased expression of the common T cell marker CD3 and of CD4/CD8 subpopulations. As reported in mammals, the *CD4* gene showed higher transcript values with respect to *CD8* [27,28] and could represent in fish an analogue of brain-resident regulatory T cell population involved in homeostasis and disease [14]. The virus-induced high expression of *TcR β* was expected and in line with values from the other T cell-related genes, and a minor contribution of *TcR γ* -related gene transcription was not surprising, being this gene mostly expressed in fish mucosal tissues. As a comparison, the importance of *TcR $\alpha\beta$* rearrangement in the mouse brain has been previously investigated and ascribed to T cell-related protection during viral infections [40,41].

By considering that viral infection of the brain induces inflammation and encephalitis, some genes coding for antiviral proteins (*Mx*, *ISG-12*) and proinflammatory cytokines (*TNFA*, *IL-1 β*) were investigated (Fig. 8). With the exclusion of *IL-1 β* , whose gene expression remained unchanged, the other genes were upregulated at a very high extent and, in agreement with innate immune responses kinetics, were higher at 3 dpi than at 6 dpi. Our results confirm and extend previous observations on RGNNV-induced gene expression increase specifically associated with sea bass antiviral and cytokine responses [18,42].

Finally, we investigated the expression of other genes we believed related to T cells and CD45-cells activities, namely *IL-2*, *IL-10*, *IFN γ* , *RAG-1* and *CD45* (Fig. 9). All these considered genes were upregulated by virus infection and thus in agreement with the data we obtained by IIF and IHC. In particular, CD45 and IFN γ are markers and effectors of glial cells [43,44] and their increase can be also related to the impressive increase of immunoreactive cells observed by IHC/qIHC. Indeed, this consideration aligns with the presumed activities attributed to glial cells in the mammalian nervous system, which are characterized by the presence of CD45 as a surface marker. These cells are known to be involved in tissue surveillance and they initiate an inflammatory response upon detecting danger signals [43]. The *IL-2* and *IL-10*, together with *TNFA*, could represent a fish counterpart of the protective system regulated by these cytokines against viral-induced encephalitis, as reported in mice [45]. It is of particular interest the presence of transcripts coding for the *RAG-1* gene in sea bass brain and its upregulation following viral infection. In mammals, the *RAG-1* presence in the CNS has been demonstrated and related to impaired behavior [46], but the knowledge on the *RAG-1*-CNS relationship is scarce. Our results suggest the CNS as a site where somatic recombination of lymphocytes occurs in response to viral infection. This speculation is supported by the observed increase in TcR β expression, which likely represents antigen-induced rearrangement of TcR sequences. Data from recent findings [4] and our work confirm relationships between the brain and retina in antiviral defense mechanisms and suggest these tissues as a target of research in CNS-associated immune activities. Future work will

be dedicated to exploring this further.

In conclusion, our research has unveiled the existence of unique immune features comprising T cells and CD45 leukocyte populations within the sea bass eye-brain axis. Such immune cells, upon viral infection, exhibit a substantial increment in the retina and brain parenchyma, accompanied by achievable *in situ* brain lymphocyte somatic recombination. The development of a CNS-immune system and eye/brain antiviral responses in vertebrates can therefore be traced back to 200 million years ago. Overall, the European sea bass emerges as a valuable model for investigating the role of brain resident and recruited immune cells in regulating CNS response to infections of vertebrates.

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Ethics

Fish trials were accomplished according to the Bioethics Committee of the University of Malaga and the Junta de Andalucía Government (Ref. n°: May 04, 2021/072). As well as by the European Union guidelines for the handling of laboratory animals (Directive 2010/63/UE) and the Spanish directive (RD53/2013) in order to minimize fish suffering.

CRediT authorship contribution statement

Valeria Pianese: conceived and supervised the project, Formal analysis, wrote the manuscript. All authors have read and agreed to the submitted version of the manuscript. **Daniel Alvarez-Torres:** and **Juan Gomez-Mata:** and **Esther Garcia-Rosado:** and **Patricia Moreno:** were responsible for fish maintenance, virus production and infection. **Anna Maria Fausto:** performed IIF and IEM. **Anna Rita Taddei:** performed IIF and IEM. **Simona Picchietti:** conceived and supervised the project, wrote the manuscript. All authors have read and agreed to the submitted version of the manuscript. **Giuseppe Scapigliati:** conceived and supervised the project, wrote the manuscript. All authors have read and agreed to the submitted version of the manuscript.

Declaration of competing interest

Authors declare there are no competing financial interests in relation to the work described.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.fsi.2024.109646>.

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